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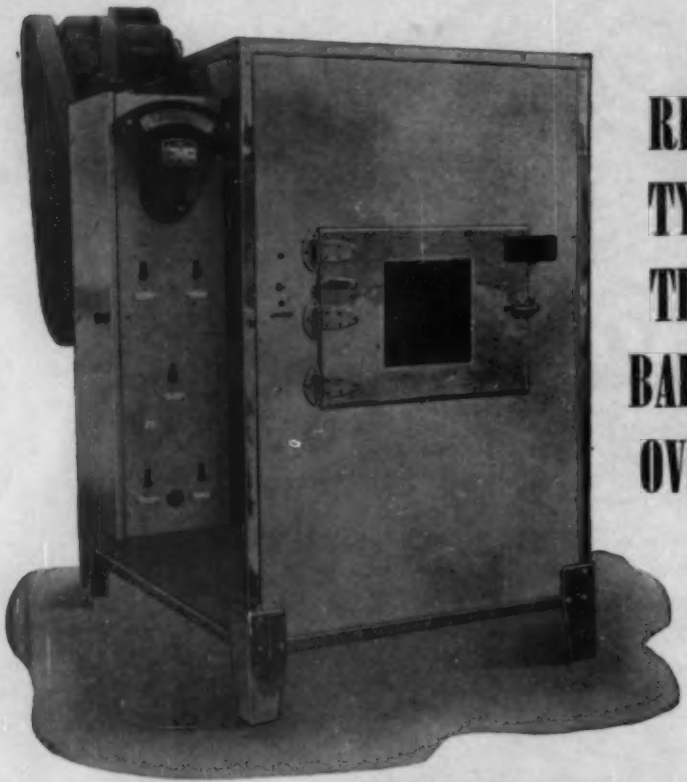

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announce the appointment of Dr. Kenneth A. Gilles as *Editor* of CEREAL CHEMISTRY to succeed the late W. F. Geddes. This appointment is effective immediately.

In the future, all editorial correspondence should be directed to: *Editor* of CEREAL CHEMISTRY, Dept. of Cereal Technology, North Dakota State University, Fargo, North Dakota.

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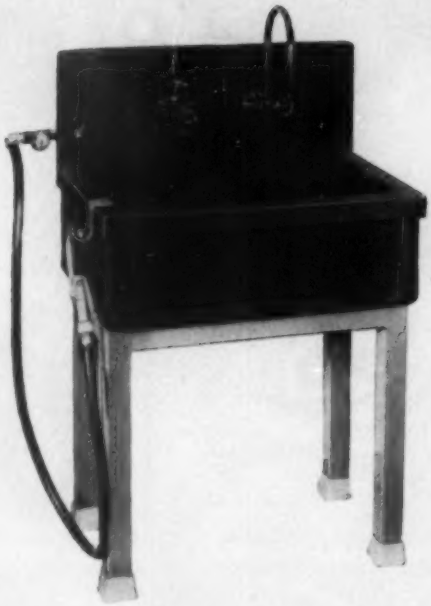

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CEREAL CHEMISTRY

VOL. 38 MAY, 1961 NO. 3

PREPARATION AND PROPERTIES OF GALACTOSYLISOMALTOL AND ISOMALTOL[1]

J. E. HODGE AND E. C. NELSON

ABSTRACT

Isomaltol, an enolic compound ($C_6H_6O_3$), first isolated in trace amounts by Backe in 1910 from a steam distillate of bread, is now prepared from lactose in 25% over-all yield. Dehydration of the glucose moiety of lactose by reaction with secondary amine salt in tertiary amine-buffered alcoholic medium yields the new compound, isomaltol beta-D-galactoside, which is hydrolyzed or pyrolyzed to produce Backe's isomaltol. Reaction of maltose with piperidine acetate under the same conditions yields 1-deoxy-1-piperidino-maltulose. This new Amadori compound does not dehydrate to a glucoside of isomaltol analogous to the lactose reaction.

Comparison of the properties of isomaltol with those of maltol under identical conditions shows that isomaltol is not a pyrone but probably a furan derivative. Isomaltol and maltol probably are formed in bread by Maillard-type browning reactions, and not by fermentation, as Backe assumed.

Maltol and isomaltol are crystalline enolic compounds, $C_6H_6O_3$, volatile with steam and readily sublimed, that have been isolated from baked cereals, bread, and bread crusts (2,3,6,30). Both compounds are reported as trace constituents of bread; however, they may contribute to the fragrant aroma of freshly baked bread and to the final flavor of bread (15). Maltol is also obtained in low yields by extracting larch bark (25,32) or fir needles (10); pyrolyzing cellulose, starch, or wood (8,11); roasting malt (5,18), chicory (28), or coffee (2,30); heating milk (21,23,24,26); autoclaving lactose or maltose with glycine (22); and degrading streptomycin with alkali (29). Whereas considerable literature exists on maltol, isomaltol has been reported by only one investigator (2,3).

Backe extracted isomaltol from a bread distillate in 1910. Nestlé's "farine lactée," a powdered preparation of condensed milk and flour,

[1]Manuscript received July 29, 1960. Contribution from the Northern Regional Research Laboratory, Peoria, Illinois. This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Presented in part at the 45th annual meeting, Chicago, Illinois, May 1960.

Note added in proof: Galactosylisomaltol now fully named 2-acetyl-3-β-D-galactopyranosyloxyfuran.

had been condemned at the customhouse in Rio de Janeiro because extracts gave a red-violet color with ferric chloride, and this was interpreted as showing the presence of salicylic acid preservative in the biscuit powder (2). Backe proved that the color was caused by a new compound, melting at 98°C., which he steam-distilled from an acid suspension of the biscuit powder (0.07 g. from 1 kg.). Lack of a suitable preparative method hindered Backe's investigations, but he did prepare three crystalline derivatives and found that his compound was isomeric with maltol (I, Fig. 1) and had some properties closely resembling it. With meager evidence, some of it in contradiction, he proposed the 3-hydroxy-5-methyl-4-pyrone structure (II, Fig. 1) for isomaltol.

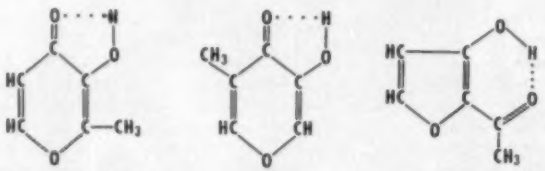


Fig. 1. Accepted structure for maltol (I) and proposed structures for isomaltol (II and III).

Results and Discussion

The present work shows that a simple, chemical dehydration of lactose by piperidine acetate (or other secondary amine salts) produces the *O*-galactoside of isomaltol in 35–40% yield. Isomaltol is then dry-distilled from the galactoside in 68% yield. Identity with Backe's isomaltol is established by preparing the same *O*-methyl ether, *O*-benzoyl ester, and moss-green copper salt that Backe reported. Discovery of this preparative method makes isomaltol available in quantity for flavor research and structural studies.

The galactoside of isomaltol, a new compound, is characterized by optical rotation and facile hydrolysis with almond emulsin at 25°C. as isomaltol beta-D-galactopyranoside. Alpha-D-Galactopyranose was isolated after both enzymatic and acid hydrolysis. Methanolysis gave methyl beta-D-galactopyranoside. Ring structure in the galactosyl radical is shown by formation of only a tetra-*O*-acetyl derivative upon acetylation of the galactoside in excess pyridine-acetic anhydride, together with the demonstration that the tetra-acetate readily forms the same *p*-nitrophenylhydrazone which is formed by acetylation of the preformed *p*-nitrophenylhydrazone of the galactoside. The mild reaction

conditions which dehydrate the glucose moiety of the disaccharide are assumed to leave the galactose moiety intact in pyranose form. Also, galactose probably remains linked to the fourth carbon atom of the glucose radical; otherwise, piperidino-hexose-reductone (35) would be formed as the major product from an unsubstituted glucose or galactose under these reaction conditions (14, 16,17).

Hydrolysis of the galactoside proceeds with mild catalysts, such as 20% acetic acid or 5% sodium bicarbonate, indicating enolic linkage of the galactosyl radical to the hydroxyl group of isomaltol. Moreover, a positive test for enol is obtained immediately after the addition of almond emulsin to an aqueous solution of the galactoside, but not before. One oxygen atom in the isomaltol radical, $C_6H_6O_3$, is therefore enolic, the second is carbonyl (formation of a *p*-nitrophenylhydrazone), and the third is not acylated in pyridine-acetic anhydride or pyridine-benzoyl chloride.

The molecular absorption coefficient (E_m, liters $mole^{-1}$ cm^{-1}) of *O*-galactosylisomaltol in methanol is 18,300 at 276 mμ. Isomaltol in methanol gives E_m 16,000 at 280 mμ, and *O*-methyl isomaltol in methanol gives 17,400 at 281 mμ. The isomaltol radical in the galactoside therefore contains as much conjugated unsaturation as is present in

TABLE I

CHARACTERISTIC DIFFERENCES IN PROPERTIES OF ISOMALTOL AND MALTOL

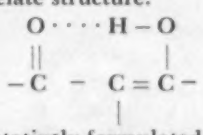
PROPERTY OR REAGENT	ISOMALTOL	MALTOL
Melting point	101° ± 2°C	162°C
Ultraviolet absorbance:		
λ_{max}; $E^{1\%}_{1\,cm}$ in 0.1*N* HCl	283 mμ; 1,366	274 mμ; 731
in 0.1*N* NaOH	318 mμ; 1,485	318 mμ; 754
IR absorbance in KBr disks:	3.23–3.43 μ;	3.11 μ (sharp)
O — H . . . O	3.50–3.77 μ[a]	
"Carbonyl"	6.20–6.45 μ[b]	6.03 μ (sharp)
Acidity:		
pH of 0.5% solution, 22°C	3.7	5.3
5% Sodium bicarbonate	CO_2 liberated	No CO_2
Diazomethane in ether, methylation	Fast, complete	Slow, incomplete
Concentrated hydrochloric acid	Decomposes[c]	Forms stable salt
Reducing power in alkali:		
Fehling solution at 100°C	Slow	Rapid
2,3,5-Triphenyltetrazolium chloride, 60°C., 5 minutes	Colorless	Red formazan
Hydrazone formation:		
p-Nitrophenyl (mono)	Forms on heating in neutral MeOH	Does not form
2,4-Dinitrophenyl (mono)	Forms readily in the cold	Does not form

[a] Two groups of rather strong, irregular, broad bands.
[b] The carbonyl band at 6.2 μ is merged with an adjacent strong band attributed to — C = C — in chelate conjugation with the carbonyl group (12).
[c] With formation of unidentified orange crystalline compound of higher molecular weight; m.p. 124°C.

isomaltol itself and in the *O*-methyl ether. Two double bonds in conjugation with the carbonyl group are indicated. For example, an isomer, 5-(hydroxymethyl)-2-furaldehyde, gives E_m 16,700 at 284 $m\mu$ (31).

Whereas Backe emphasized the similar properties of isomaltol and maltol, this work shows their similar properties (general solubilities, ferric chloride color reaction, iodoform reaction, and *C*-methyl analysis) are less important for structural considerations than their dissimilar properties. Important dissimilarities noted in parallel comparisons of physical and chemical properties appear in Table I.

Isomaltol is more volatile, acidic, ketonic, unstable to acid, and stable to alkali than maltol. These significant differences (Table I) suggest that isomaltol does not have a pyrone ring structure. From the acid-lability, alkali-stability characteristics, a 2-substituted furan structure is indicated. Pyrones do not form hydrazones by the usual methods. Not only does isomaltol readily form *p*-nitrophenyl- and 2,4-dinitrophenylhydrazones, but the *O*-methyl enol ether derivative also forms a *p*-nitrophenylhydrazone under neutral conditions. This demonstration of distinctly separate carbonyl and enolic hydroxyl functions, together with high volatility, displaced infrared bands for O − H · · · O and carbonyl (12,20,27); formation of a green copper-chelate salt soluble in organic solvents and insoluble in water (compare acetylacetone, acetoacetic ester) allows the assumption that isomaltol contains an enolized beta-diketone chelate structure:



Therefore isomaltol is tentatively formulated as III (Fig. 1), with work toward proof of structure still in progress.

Backe conducted many experiments to show the mode of isomaltol formation in bread. His biscuit powder, ether-extracted before baking, gave maltol, not isomaltol. After baking and steam-distilling an acidified suspension of the bread, he extracted much more isomaltol than maltol from the distillate. Ordinary wheat flour, heated at 150°C. without water, gave no isomaltol or maltol according to the ferric chloride test for enol on an ether extract of the flour. When the wheat flour was worked into a dough and heated at 150°C. in the same way, the ether extract gave a strong purple color with ferric chloride, which could have been caused by the presence of either isomaltol or maltol, or both. When the "dry" wheat flour was first heated at 150°C., then worked into a dough and rebaked for 2 hours at 150°C., the color reaction was weak; but when the preheated, enzyme-inactive flour was treated with

malt extract, then worked into a dough and rebaked, the color reaction was very strong. The malt extract alone, dried on an inert material, gave no color reaction after baking. Starch gave negative tests when heated dry and as a paste, but when pasted with malt extract before heating, the test was strongly positive. On the other hand, starch treated with a purified commercial diastate (alcohol precipitate of malt extract) gave no color reaction under the same conditions. Maltose (that contained some malt impurities) and pure sucrose gave the color reaction when heated with water alone at 150°C. in a sealed tube, but glucose did not. Backe concluded that wheat flour and malt, but not diastase, contained a "special enzyme" that, along with heat, is necessary for the formation of isomaltol in bread.

Patton and co-workers showed the presence of maltol in overheated skimmilk (21,23) and also in milk evaporated in the usual way (26). Milk containing lactose-l-C^{14} gave radioactive maltol upon evaporation (23,24). In model systems, lactose and maltose gave maltol upon autoclaving with glycine at pH 7, but glucose and galactose (also sucrose, starch, and cellulose) did not (22). Patton's work allows an interpretation of Backe's findings. Maltol present in the biscuit powder before baking was preformed in the condensed milk fraction. However, some of the maltol remaining after baking could generate from maltose (liberated by diastatic action in the dough) by its interaction with amino acids, amine salts, and peptides in Maillard-type browning reactions (13).

Patton and Flipse showed that maltol was generated from the glucose moiety of lactose in milk (24). Our work shows that isomaltol also is generated from the glucose moiety of lactose by interaction with secondary amine salts. In another analogy with Patton's work, our experimental conditions do not produce isomaltol from hexoses; crystalline amino-hexose-reductones are isolated instead (14,16). In contrast with Patton's experiments, *O*-glucosylisomaltol was not obtained from several maltose-piperidine acetate reactions under conditions that gave *O*-galactosylisomaltol from lactose. Instead, the new Amadori rearrangement product, l-deoxy-l-piperidino-maltulose crystallized out, and this surprisingly stable compound would not yield the isomaltol radical upon further heating with piperidine acetate.

It is now more reasonable to assume that maltol and isomaltol are formed nonenzymatically from maltose and/or lactose in bread. The amine reagent in Backe's experiments would be the free amino acids and peptides present in the malt extract which, however, would not likely be present in the precipitated diastase he used. Free amino acids (33) and the amino groups of peptides and proteins in doughs probably

act on the disaccharides in Maillard-type browning reactions during baking.

More research is needed to establish the presence, concentrations, and mode of formation of maltol and isomaltol in bread. Backe's claims for isomaltol (2) and Sherman's for maltol (30) in *ordinary* wheat breads should be substantiated; both men used only ferric chloride color reactions that do not distinguish between the two. Further investigations are needed because both compounds possibly contribute to bread flavor (15).

Experimental

Melting points, determined in capillary tubes, are corrected. Chemicals used are reagent grade or the best commercial grade unless otherwise specified.

O-Galactosylisomaltol. In a 2-liter, three-necked reaction flask, fitted with anchor-blade stirrer, reflux condenser, dropping funnel, and hemispherical electric heating mantle, 1 mole of alpha-lactose hydrate (360 g.) is stirred into 300 ml. absolute ethanol at room temperature until smoothly dispersed. One mole of piperidine (100 ml. practical grade) is added; then 1 mole of glacial acetic acid (58 ml.) is dropped, over several minutes, into the continually stirred mixture. After the dropping funnel is replaced by a thermometer, the reaction temperature is held at 78° ± 2°C. Triethylamine (50 ml.) is added to keep the reaction mixture alkaline over the first 12 hours. When solution occurs after 10 to 12 hours, more triethylamine (50 ml.) is added, and heating at 78°C. is continued for a total of 24 hours[2]. If the crystalline product does not separate spontaneously during the heating period, seed crystals are produced by diluting a sample of the mixture with ethanol.

The heating mantle is replaced with a crushed-ice bath, ethanol (300 ml.) is added with continued stirring, and the crystalline mush is held at 5°C. or below for 1 hour before filtering with suction. The filter cake is washed with ethanol, suspended and stirred in ethanol, refiltered, pulverized, and dried in a vacuum desiccator over anhydrous calcium chloride to constant weight. The crude yield is 105–107 g., 37% of theory; m.p. 203°–205°C., with boiling and distillation of isomaltol to the upper walls of the capillary tube. Processing of the mother liquor yields 5 to 10 g. piperidino-hexose-reductone (35) but very little more *O*-galactosylisomaltol.

The same yield of *O*-galactosylisomaltol is obtained when anhy-

[2] If the reaction mixture is boiled continuously, the temperature slowly increases from 81° to 83°C. More triethylamine is needed to buffer the mixture, and the yield is the same.

drous beta-lactose is substituted for alpha-lactose hydrate.

When the procedure is repeated with 0.75, 1.00, and 1.25 moles of piperidine acetate per mole of lactose, the yields are 25, 37, and 41% of theory based on the amount of lactose added, and 33, 37, and 33% based on piperidine, respectively. With 0.75 mole of piperidine acetate, the 24-hour product is contaminated with unchanged lactose (removed by the recrystallization from water before determining the yield). However, when the reaction time is extended to 32 hours to dissolve all the lactose, the yield is increased to 35% based on lactose and 47% based on piperidine.

When the procedure is repeated with 1 mole of morpholine instead of piperidine, except that the mixture is refluxed at 82°C. for 24 hours, the yield is 21% and is increased to 26% by refluxing for a second 24-hour period.

With diethanolamine in place of piperidine, the yield is only 13% of theory. Monoethanolamine gives a dark, hygroscopic product that yields no isomaltol on hydrolysis. Glycine, in place of piperidine acetate, is scarcely soluble and does not react in 29 hours at 75°C. When triethylamine replaces piperidine, 91% of the lactose is recovered unchanged. When sterically hindered di-isopropylamine is used instead of triethylamine as the nonreactant buffer, the yield does not change.

One mole of anhydrous dimethylamine acetate, 1 mole alpha-lactose hydrate, 100 g. trimethylamine, and 500 ml. methanol when refluxed at the boiling point (60° to 72°C.) for 24 hours yields 21% of the theoretical amount of *O*-galactosylisomaltol. Reheating the mixture at 76°–78°C. for 8 hours increases the yield to only 22% of theory, and 8 g. of dimethylamino-hexose-reductone (16) is recovered from the filtrate. When dimethylamine acetate is used in dimethylformamide solvent with heating at 90°C. for 15 hours and with vacuum distillation of solvent during the last 3 hours, the yield is 18% of theory.

Recrystallization of the crude products from methanol yields analytically pure *O*-galactosylisomaltol that melts and decomposes at 204°–205°C. Recrystallization from water yields a bulky, hydrous precipitate that melts at about 195°C. when dry. However, this low-melting form gives the same specific optical rotation, $[\alpha]_D^{25}$ −4.5° ($c = 2$, $l = 2$, in water) and the same crystalline tetra-*O*-acetyl derivative as the more dense high-melting form. Calculated for $C_{12}H_{16}O_8$: 50.00% C, 5.60% H, 288.3 molecular weight. Found: 50.17% C, 5.63% H, 0.00% N, 0.96% *C*-methyl per 288.3 molecular weight (4).

The ultraviolet absorption spectrum between 220 and 400 mμ shows a single, strong maximum at 278 mμ, E (1%, 1 cm.) = 632, in

water; at 276 mμ, E = 634, in methanol. The infrared spectrum of either methanol- or water-recrystallized forms in potassium bromide disks shows no absorption bands in the range 3.5 to 6.0 μ. A very strong absorption appears at 6.11 μ (1,637 cm^{-1}) with a shoulder (strong) at 6.06 μ (1,650 cm^{-1}). Other strong bands between 6 and 7 μ are placed at 6.28 (1,592 cm^{-1}) and 6.83 μ (1,464 cm^{-1}) with a medium band at 6.70 μ (1,493 cm^{-1}).

O-Galactosylisomaltol is neutral, moderately soluble in water (about 3 g. per 100 ml. at 25°C.), and sparingly soluble in ice water or neutral organic solvents at room temperature. It is moderately soluble in pyridine, *N,N*-dimethylformamide, and boiling methanol. It does not reduce 2,6-dichlorophenolindophenol in 0.1*N* sodium hydroxide at 25°C., but it does reduce hot Fehling solution. It gives no deep color with ferric chloride in aqueous or alcoholic solution.

Hydrolysis and Methanolysis of O-*Galactosylisomaltol.* Fifty millimoles of *O*-galactosylisomaltol (14.4 g.) in 200 ml. of 2*M* orthophosphoric acid are steam distilled. One liter of distillate is collected and exhaustively extracted with chloroform until a negative test for enol is obtained in the aqueous layer. Concentration of the combined, dried chloroform extracts, with trituration of the distillation residue in 1:1 ether-petroleum ether, yields 2.5 g. crude isomaltol, 41% of theory. Recrystallization from benzene in three crops gives 2.2 g. of pure isomaltol, m.p. 98°–98.5°C., agreeing in all properties with those reported by Backe (3). Sublimation at 1 mm. mercury pressure gives white, opaque crystals, m.p. 100°–101°C. Calculated for $C_6H_6O_3$: 57.14% C, 4.80% H, 126.1 molecular weight. Found: 57.18% C, 4.80% H, 124 neutral equivalent, 0.98 *C*-methyl per 126.1 molecular weight (4).

A solution of 1.7 g. *O*-galactosylisomaltol in 20% aqueous acetic acid is refluxed for 8 hours and distilled at atmospheric pressure for 4 hours. Dilution of the still-pot residue with alcohol and ether gives, on long standing at 1°C., 0.54 g. (51% of theory) of reducing sugar identified as alpha-D-galactose by optical rotation $[\alpha]_D^{25}$ + 150° (initial, by extrapolation) → + 78.7° (final, $c = 3.0$, $l = 1$, in water); by melting point, 165°–167°C.; and by paper chromatography parallel with an authentic sample.

A solution of *O*-galactosylisomaltol in 5% sodium bicarbonate, heated at 100° for 20 minutes and then neutralized, gives a deep purple with a drop of ferric chloride solution.

O-Galactosylisomaltol (2.88 g.) in 100 ml. water is treated with 75 mg. almond emulsin and stored at 25°C. Immediately after addition of the emulsin, a sample of the solution gives a deep purple color with

a drop of ferric chloride solution. After 1 day, the mixture is extracted with 250 ml. ether, and on four successive days thereafter with 150 ml. ether. The combined extracts, dried and evaporated, gave a crude crystalline residue which, when dissolved in dry ether and cooled to −17°C., deposited 1.0 g. (79% of theory) of crystalline isomaltol. The aqueous phase remaining after the ether extractions gave (in three crops upon evaporation and dilution with ethanol) 78% of the theoretical amount of crystalline alpha-D-galactose, identified by melting point, optical rotation, and paper chromatography parallel with an authentic sample.

Methanolysis of *O*-galactosylisomaltol in anhydrous methanol containing 1.1% (wt/vol) hydrogen chloride for 5 days at 25°C. produced 74% of the theoretical amount of crude methyl beta-D-galactopyranoside. After two recrystallizations from methanol, the melting point was 177°–177.5°C. Calculated for $C_7H_{14}O_6$: 43.30% C; 7.28% H. Found: 43.30% C, 7.33% H.

O-*Galactosylisomaltol Tetra-Acetate.* Fifty millimoles (14.4 g.) of *O*-galactosylisomaltol are suspended in 75 ml. pyridine and 30 ml. acetic anhydride (300 mmoles). After standing 40 hours at 25°C., the yellow solution is filtered and poured into 600 ml. ice water; yield, 21.7 g., 95% of theory, m.p. 127°–128°C. Recrystallization from 150 ml. ethanol gives 21.5 g. of glittering crystals; m.p. 128.5°–129.5°C.; $[\alpha]_D^{25}$ − 14.6° ($c = 5.0$, $l = 2$, in chloroform). Calculated for $C_{20}H_{24}O_{12}$: 52.63% C, 5.30% H, 37.73% $COCH_3$. Found: 52.81% C, 5.31% H, 37.53% $COCH_3$ by method of Kunz (19).

O-*Galactosylisomaltol* p-*Nitrophenylhydrazone.* Methanol solutions of 6.7 mmoles *p*-nitrophenylhydrazine and 5.0 mmoles *O*-galactosylisomaltol are combined and refluxed for 2 hours on the steam pot. The deep red hydrazone is filtered off, extracted several times with boiling methanol, and dried under vacuum at 100°C.; yield 1.4 g., 65% of theory; m.p. 217°–218°C. with decomposition. Calculated for $C_{18}H_{21}N_3O_9$: 51.06% C, 5.00% H, 9.93% N. Found: 50.6% C, 5.11% H, 9.90% N (Dumas).

O-*Galactosylisomaltol* p-*Nitrophenylhydrazone Tetra-Acetate.* Acetylation of *O*-galactosylisomaltol *p*-nitrophenylhydrazone in pyridine-acetic anhydride in the usual way gives a mixture, the major component of which is the orange-yellow tetra-*O*-acetyl derivative, m.p. 143.5°–144.5°C. Calculated for $C_{26}H_{29}N_3O_{13}$: 52.79% C, 4.94% H, 7.10% N. Found: 52.3% C, 5.05% H, 6.98% N (Dumas).

On treating the tetra-*O*-acetyl derivative of *O*-galactosylisomaltol with *p*-nitrophenylhydrazine acetate in methanol, the same compound, m.p. 143.5°–144.5°C. (mixed melting point unchanged) was obtained.

Isomaltol. Crude *O*-galactosylisomaltol (40 g.) is placed in an alembic flask constructed with annular receiver immediately above the spherical cucurbit of 250-ml. capacity. Carbon dioxide is slowly passed through a side-arm in the cucurbit; then the flask is lowered into a preheated Wood's metal bath at 240°C. Distillation of isomaltol begins with caramelization of the galactoside and continues for 10 minutes at atmospheric pressure with bath temperature in the range 240°–250°C. The delivery arm is irradiated with a heat lamp to keep the distillate liquid until it drops into the cooled receiver. Crystallization occurs immediately. The crude cake is broken up under 15 ml. water, and the dispersion is cooled to 2°C., after which it is filtered and the filter cake is washed with ice water. After drying over anhydrous calcium chloride at atmospheric pressure, 12 g. of pale-yellow crystalline powder (68% of theory) melting at 101°–102°C. with sublimation are obtained. Recrystallization from water or ether gives the pure compound, which melts rather sharply in the range 98°–103°C. The melting point varies with crystalline form and density of packing in the capillary tube. The compound is identical with isomaltol obtained by hydrolysis (analysis above).

Isomaltol is optically inactive. In the ultraviolet, a single absorption band appears at 280 mμ, E (1%, 1 cm.) = 1,270 in absolute methanol. In 0.1*N* hydrochloric acid, the maximum is at 283 mμ, E = 1,366. In 0.1*N* sodium hydroxide, the major peak shifts to 318 mμ, E = 1,485, and a minor peak becomes visible at 258 mμ, E = 500.

Isomaltol is soluble in alcohols, acetone, ether, chloroform, benzene, ethyl acetate, and hot water; it is difficultly soluble in petroleum ether and cold water. Aqueous solutions are acidic (pH 3.60 at 25°C., $c = 1.00$); they liberate carbon dioxide from 5% sodium bicarbonate and titrate to a definite end point at pH 8.7 with sodium hydroxide. Aerated neutralized and weakly alkaline solutions are yellow; these turn colorless upon acidification. Isomaltol can be recovered from heated 5*N* sodium hydroxide solutions in high yield if air is excluded. In contrast, strong acids readily decompose isomaltol. Hot 25% aqueous *p*-toluenesulfonic acid in the Elek-Harte acetyl determination (7) liberates steam-volatile acid that is not unaltered isomaltol. The distillate contains 34.4% acetyl equivalent by titration with base (7) and 3.3% formyl equivalent by bromine oxidation (1). Cold, concentrated hydrochloric or phosphoric acid solutions of isomaltol turn yellow, red, and deep greenish brown before depositing a crystalline precipitate. Recrystallization of the precipitate gives bright-orange crystals, yet unidentified, that melt sharply at 124°C. Calculated for $C_{12}H_{10}O_5$: 61.53% C, 4.30% H, 234.2 molecular weight. Calculated for

$C_{10}H_8O_4$: 62.50% C, 4.20% H, 192 molecular weight. Found: 62.42% C, 4.31% H, 209 molecular weight (Rast), 0.89 *C*-methyl per 192 molecular weight (4).

Aqueous solutions of isomaltol at pH 3.5 reduce permanganate readily and decolorize small amounts of 2,6-dichlorophenolindophenol quickly in the cold. With the latter reagent, turbidity soon develops, and unreduced red dye is later adsorbed on the initially colorless precipitate. Isomaltol does not reduce 2,6-dichlorophenolindophenol in 0.1 to 1.0*N* sodium hydroxide solutions at 20°C., but reduction occurs slowly upon heating. Also, Tollens reagent is not reduced even upon warming to 50°C. Fehling solution is slowly reduced upon heating at 100°C.

Highly dilute aqueous or alcoholic solutions of isomaltol give stable purple colors with ferric chloride. The hue changes with pH and is virtually the same for maltol and isomaltol at the same pH – violet at pH 3, cherry-red at pH 6. Titanium trichloride gives deep violet in methanol; however, in the Weygand-Csendes test in 95% methanol-pyridine (3:1), upon shaking with air, the initial pure blue lightens to greenish blue and then becomes a light orange-red; on standing, a bright yellow precipitate forms (34).

Isomaltol-O-Methyl Ether. A cold solution of 1.4 g. diazomethane in 50 ml. ether is added, 5 ml. at a time over 10 minutes, to 50 ml. of a cold ether solution containing 3.6 g. isomaltol. The methylated product soon crystallizes. The mixture is stored at 2°C. for several hours, the supernatant solution is decanted, and the crystalline precipitate is washed with cold ether and collected on a filter and dried (1.65 g.). A second crop (0.75 g.) is obtained by concentrating the decanted solution to 20 ml. volume, cooling, and filtering. A third, reddish crop (0.30 g.) separates from the filtrate after dilution with light petroleum ether and cooling. The total yield is 2.70 g., 68% of theory. Recrystallization from ether or water gives hard, dense, colorless prisms, m.p. 101.5°–103°C., that sublime on further heating. Backe reported a melting point of 101°–102°C. for isomaltol-*O*-methyl ether (3). The aqueous solution is neutral and gives no trace of purple with ferric chloride. Calculated for $C_7H_8O_3$: 60.00% C, 5.75% H, 22.15% OCH_3, 140.1 molecular weight. Found: 59.89% C, 5.75% H, 22.59% OCH_3, 154 molecular weight (Rast).

Isomaltol-*O*-methyl ether in methanol gives an absorption maximum at 281 mμ, E (1%, 1 cm. = 1,240, E_m = 17,400) that does not shift in acid or alkali. In carbon tetrachloride, no significant bands appeared in the infrared below 3.3 μ (above 3030 cm.$^{-1}$). A very strong band at 5.92–5.94 μ (1687 cm.$^{-1}$) corresponds to the carbonyl group.

Isomaltol O-Methyl Ether p-Nitrophenylhydrazone. Isomaltol-*O*-methyl ether (60 mg.) in 1 ml. warm methanol, *p*-nitrophenylhydrazine (70 mg.) in 2.5 ml. warm methanol, and 15 drops of 3*N* acetic acid are mixed. After boiling 4 minutes, the combined solution turns red. On cooling and standing, deep red needles (45 mg.) separate, m.p. 176°–177.5°C. The same compound is produced, without acetic acid, by boiling the alkaline solution of methyl ether and hydrazine for 35 minutes. Calculated for $C_{13}H_{13}N_3O_4$: 56.72% C, 4.76% H, 15.27% N, 11.27% OCH_3. Found: 56.81% C, 4.80% H, 15.53% N (Dumas), 11.66% OCH_3.

Isomaltol O-Benzoyl Ester. Isomaltol (0.21 g.) in 2 ml. dry pyridine is added to a cold mixture of benzoyl chloride (0.4 g.) in 3 ml. dry pyridine. The clear solution is allowed to stand at 25°C. for 18 hours before it is poured into 50 ml. of ice water. The crystalline precipitate (0.26 g.) melts at 98°–99°C. without sublimation on further heating to 200°C. After recrystallization from 1:1 benzene-toluene, the benzoate melts at 100°–101°C. and gives no trace of purple with ferric chloride in 95% ethanol. Calculated for $C_{13}H_{10}O_4$: 67.82% C, 4.38% H. Found: 67.90% C, 4.36% H. Backe reported a melting point of 99°C. for the benzoate of isomaltol (3).

Copper Salt of Isomaltol. A solution of 1.00 millimole copper sulfate is added to 0.95 millimole isomaltol in 20 ml. water. The translucent, hydrated green crystals that form slowly at 0°C. effloresce to an opaque, dull green powder when dried in air. No decomposition is evident on heating to 200°C. Calculated for Cu $(C_6H_5O_3)_2$: 45.94% C, 3.21% H. Found: 45.93% C, 3.29% H.

The moss-green copper salt is insoluble in water, soluble in ammonia (deep blue) and glycine solutions (blue), and in methanol (green), chloroform (green), and pyridine (green). Upon addition of aqueous sodium hydroxide to the green methanol solution, the color changes to blue as cupric hydroxide is precipitated. The properties of this copper salt agree wholly with those reported by Backe (3).

Isomaltol p-Nitrophenylhydrazone. A solution of isomaltol (0.64 g., 5.0 mmoles) in 5 ml. methanol is added to a solution of 0.93 g. (6.0 mmoles) *p*-nitrophenylhydrazine in 30 ml. hot methanol. The weakly alkaline solution is heated at the boiling point for 1 hour and allowed to stand at 25°C. overnight. The first crop, recrystallized twice from nitrobenzene, yields 15 mg. of a slightly soluble, orange-red compound that melts at 287°–289°C. Other crops isolated from the filtrate, 0.75 g., melt at 181°–183°C. Recrystallization from nitrobenzene yields a deep red monohydrazone, melting at 187°–188°C. with decomposition. Calculated for $C_{12}H_{11}N_3O_4$: 55.17% C, 4.24% H, 16.09% N. Found:

55.15% C, 4.14% H, 16.9% N (Dumas).

Preparation with addition of acetic acid in the usual way gave the same hydrazone in a shorter reaction time.

Isomaltol 2,4-Dinitrophenylhydrazone. A solution of isomaltol (0.21 g., 1.67 mmoles) in 10 ml. ethanol is added to a solution of 0.40 g. (2.0 mmoles) of 2,4-dinitrophenylhydrazine in 2 ml. concentrated sulfuric acid, 3 ml. water, 10 ml. ethanol. Crystallization begins after 4 minutes at 25°C., yielding 0.44 g. (86% of theory) of deep-red hydrazone. Recrystallization from ethanol-ethyl acetate yields the pure monohydrazone melting at 216°C. with decomposition. Calculated for $C_{12}H_{10}N_4O_6$: 47.06% C, 3.29% H, 18.30% N. Found: 47.0% C, 3.28% H, 19.18% N (Dumas).

1-Deoxy-1-Piperidino-Maltulose. When beta-maltose hydrate was substituted for alpha-lactose hydrate in the procedure to prepare *O*-galactosylisomaltol, an intermediate compound crystallized and remained nearly insoluble over 12 hours of heating at 78°–80°C. This intermediate, identified as the Amadori rearrangement product, is prepared in higher yield as follows:

Maltose hydrate, 45.0 g. (0.125 mole), is mechanically stirred in 37 ml. absolute ethanol and 13 ml. triethylamine while 21.3 g. (0.25 mole) of piperidine (practical grade) and 15 g. (0.25 mole) of glacial acetic acid are added dropwise to the mixture. A heating mantle is applied to provide constant reaction temperature of 75°C. The mixture darkens, and all maltose is dissolved after 20 minutes. After 1 hour, crystallization of the product begins. After 2 hours at 75°C., the reaction is stopped by adding 100 ml. absolute ethanol and cooling in an ice water bath for 1 hour. The crystalline product is filtered with suction, washed until white with ethanol, and dried in a vacuum desiccator over calcium chloride. Yield, 33.3 g., 66% of theory; melting point, 173°C., with decomposition. A sample recrystallized from 100 parts boiling methanol melted and decomposed sharply at 174°C. and gave specific optical rotation $[\alpha]_D^{25} + 48.0°$ ($c = 0.5$, $l = 4$, pH 10.1 in water). Calculated for $C_{17}H_{31}NO_{10}$: 49.87% C, 7.63% H, 3.42% N. Found: 49.9% C, 7.69% H, 3.43% N.

The compound is strongly basic and nearly insoluble in organic solvents. It reduces 2,6-dichlorophenolindophenol (also 2,3,5-triphenyltetrazolium chloride) quickly in 0.1*N* sodium hydroxide at 20°C. Also, *o*-dinitrobenzene is reduced to a purple dye under Fearon and Kawerau's ascorbic acid test conditions (9) within less than 1 minute, another distinction between Amadori compounds and *N*-glycosides (17). The aqueous solution at 23°C. ($c = 2.0$, pH 10.25) turns golden yellow on standing in the absence of air and develops reductonelike

reducing power toward indophenol, *o*-dinitrobenzene, and tetrazolium salts, like 1-deoxy-1-piperidino-D-fructose (17).

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FLAVOR OF BREAD AND PASTRY UPON ADDITION OF MALTOL, ISOMALTOL, AND GALACTOSYLISOMALTOL[1]

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ABSTRACT

A chemical method of preparing isomaltol developed recently in the Northern Laboratory has permitted for the first time extensive investigation of this compound. Similarity of the odor of isomaltol to the fragrant, caramel-like odor of maltol prompted a flavor comparison of the two compounds in aqueous and breadlike media. Both compounds have been reported as minor constituents of bread.

Taste panel results show that maltol and isomaltol give similar caramel-like flavors, sometimes described as fruity. Isomaltol is generally described as sweeter, less bitter, weaker, and, at times, less pleasant than maltol. When each is incorporated in yeast rolls at 0.1% of the flour weight, flavor difference in the breads is frequently detected. The flavor is described as similar to that of the control fresh bread, only stronger. Isomaltol is the more volatile, and much of it is lost during baking.

The β-D-galactoside of isomaltol, easily prepared from milk sugar, has a bitter taste. In fermenting doughs, it is split into isomaltol and galactose and, in some baking pastries, by heat and moisture. Pie crust that contained 0.5% *O*-galactosylisomaltol before baking was preferred by tasters over the control.

Maltol (3-hydroxy-2-methyl-4-pyrone) and isomaltol (structure not

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proved) are volatile, crystalline, enolic compounds ($C_6H_6O_3$) that have been isolated in trace amounts from baked cereals and breads (1,2,3,12). Maltol is the better known and is sold commercially as a flavoring agent. Isomaltol was discovered and isolated by Backe in 1910 (1,2) from a bread made with special flour containing condensed milk (biscuit powder[2]). Backe also demonstrated the presence of isomaltol (or maltol!) in other wheat breads by means of the characteristic purple color reaction that isomaltol (also maltol) gives with ferric chloride (1).

Patton isolated maltol from heated milk (7,11) and also from autoclaved solutions of maltose or lactose and glycine (8). Glucose or galactose did not yield maltol under the same conditions. Condensed milk containing lactose-1-C^{14} gave radioactive maltol (9,10). Hodge and Nelson isolated *O*-galactosylisomaltol and isomaltol from alcoholic solutions of lactose heated with secondary amine salts (5), but glucose, galactose, mannose, or fructose did not yield isomaltol under the same conditions (4,6). It appears that maltol and isomaltol arise from the reducing glucose radicals in maltose and/or lactose by interaction with amino groups during the baking of bread.

Backe did not report an odor for isomaltol, *per se*; he reported that isomaltol gives rise to a strong, fragrant odor on oxidation with permanganate, silver oxide, Fehling solution, or treatment with formalin. Hodge and Nelson found that pure isomaltol has a palpable odor[3]. Similarity of isomaltol odor to the fragrant, caramel-like odor of maltol prompted this comparison of their flavors in aqueous and breadlike media by a taste panel.

Materials and Methods

Materials. Isomaltol and *O*-galactosylisomaltol were prepared by the method of Hodge and Nelson (5), and each was recrystallized from water until it was colorless. The commercial product "Palatone"[4], a specially purified form of maltol, m.p. 162°C., was used.

Tests on Aqueous Solutions. Potassium permanganate was added to distilled water; the pink solution was distilled and then redistilled in all-glass apparatus. This water was aerated and used to make the maltol, isomaltol, and *O*-galactosylisomaltol solutions and as a mouth wash for the tasters. Because 0.2% isomaltol solutions at pH 3.7 are definitely sour and astringent, both maltol and isomaltol solutions were neutralized to pH 6.0 with 5% sodium bicarbonate solution. The

[2] Nestlé's food (farine lactée), a powdered preparation of wheat flour and condensed milk.

[3] A more penetrating, fragrant, phenolic, or aromatic aldehyde odor is prominent during the distillation of isomaltol when a yellow substance codistills, as in the pyrolysis of *O*-galactosylisomaltol. A similar odor is produced by permanganate oxidation and by boiling solutions of isomaltol in 20% sodium hydroxide; hence, this odor is probably the one observed by Backe (2).

[4] The mention of trade products does not imply that they are endorsed or recommended by the Department of Agriculture over similar products not mentioned.

isomaltol solutions were golden yellow at pH 6, whereas the maltol solutions were colorless. Therefore, lighting in the taste-panel room was adjusted to eliminate the color difference. Solutions were tasted under conditions which were as free as possible from extraneous distractions. Individual booths provided privacy, and temperature (25°C.) and humidity (40%) were kept constant.

Each treated sample was compared with a control at two test sessions; the treated sample was tasted first at one session and second at the other. The number of tasters at each session varied from 12 to 20, but the same group of people was used throughout the tests. Instructions were to describe odor, taste, and any difference in taste between the control and treated samples, and then to state a preference. The voluntary answers were grouped (sweet, sour, stale, flat, bitter, like fresh bread, etc.), and the responses under each group were totaled for both test sessions.

Preparation and Tests on Yeast Rolls. To conserve time and materials and to give more crust surface per unit volume, small yeast rolls were baked instead of loaf bread. Yeast rolls were prepared by the following recipe:

½ tbsp. sugar
⅛ tsp. salt
⅛ tbsp. fat (margarine)
the additive, when used
} in 2 tbsp. boiling water
1½ tbsp. egg
½ tsp. yeast in ½ tbsp. warm water
½ cup flour (56.5 g.)

The percentage of additive used in each test was based on the weight of the flour. After the first rising, the dough was made into rolls which were set to rise again until doubled in bulk (about 1 hour) and then were baked for 10 minutes at 218°C. Odor during baking was observed at the oven vents and upon first opening the oven door. Samples were presented to the taste panel in covered beakers at 55°C. under lighting controlled to obscure color differences[5].

Preparation and Tests on Pie Crust. Pie crust, prepared in the usual way from 62.5 g. wheat flour, 36.0 g. shortening (hydrogenated vegetable oil), and 15.0 ml. cold water, was mixed and rolled to ⅛-in. thickness. Cut strips were baked at 260°C. for 8 minutes. *O*-Galactosylisomaltol was added at a selected percentage of the flour weight. Selected strips of pie crust of the same degree of brownness were presented to the panel in covered beakers at 55°C.

Test for Maltol and Isomaltol in Baked Products. To determine roughly the amount of maltol or isomaltol remaining in the baked

[5] Pumpkin-pink color was noted in the rolls that contained maltol, both before and after baking.

products, 2-g. samples were pulverized under 10 ml. of 50% aqueous ethanol in test tubes. After the triturated sample had stood in the alcohol for 30 minutes, 5 ml. of the clear supernatant liquor were decanted and tested for the enolic hydroxyl group of the additive with 3 drops of 3% ferric chloride solution in 95% ethanol. In some cases, the supernatant liquor had to be decanted and centrifuged. According to the depth of color formed, concentrations were estimated as *strong, medium, weak,* or *negative.*

Results and Discussion

Tests on Aqueous Solutions. Results of flavor evaluations on isomaltol and maltol at 0.2 and 0.5% concentrations in doubly distilled water, neutralized to pH 6, are recorded in Table I. Differences in intensity of the flavors were specified by the panel as follows: maltol

TABLE I

FLAVOR OF ISOMALTOL AND MALTOL IN WATER
(Neutralized to pH 6 with sodium bicarbonate)

Flavor Descriptions	31 Tasters		20 Tasters	
	Isomaltol, 0.2%	Maltol, 0.2%	Isomaltol, 0.5%	Maltol, 0.5%
Burnt sugar, caramel	8	13	10	10
Fruity[a]	7	6	4	2
Sweet	9	0	6	1
Sour	2	3	4	6
Bitter	2	12	4	11
Stale, metallic, rusty	3	3	1	1
Malty	0	0	1	0
Salty	1	0	1	0
Flat, tasteless	2	0	1	0

[a] Fruit mentioned: apple, cherry, melon, plum, strawberry, fruit pie, and artificial fruit flavor.

has the stronger caramel-like odor and taste and is much more bitter; isomaltol is sweeter and slightly more fruity. Although both isomaltol and maltol solutions were neutralized with sodium bicarbonate, only two of fifty-one tasters thought the soda solutions were salty. Sourness was specified even though the solutions were at pH 6.

Tests on Yeast Rolls. Isomaltol and maltol, separately incorporated in yeast roll dough at 0.1% of the flour weight, imparted a fruity odor before baking and a sweeter, more intense, fresh-bread odor during baking. Isomaltol gave, in addition to the fruity-caramel, fresh-bread odor, an unpleasant overtone described as medicinal and grassy; maltol gave an entirely pleasing and stronger odor. The maltol-treated dough wetted more readily and gave slightly increased volume in the bread over the control. Color of the isomaltol-treated dough was not noticeably different from the control; however, in direct visual com-

TABLE II

FLAVOR TESTS ON YEAST ROLLS: ISOMALTOL VS. CONTROL

FLAVOR DESCRIPTIONS (34 Tasters)	CONTROL	ISOMALTOL, 0.1%
Odor		
Fresh bread	12	21
Yeasty, doughy	15	6
Stale, musty, moldy	2	4
Sweet	0	3
Taste		
Fresh bread	12	16
Sweet	3	5
Sour	3	2
Stale, musty	5	2
Salty	2	0
Cucumber	0	1
Preference (No preference: 4)	13	17
Reason: More fresh-bread flavor	8	10
Sweeter, less off-flavor	4	7

parison, the treated bread reflected less light. Maltol-treated dough was consistently and decidedly pinkish orange or pumpkin-colored. Color intensity diminished on baking, but still was quite noticeable in comparison with the control bread. Results of flavor evaluations are given in Tables II, III, and IV.

Isomaltol in yeast rolls gave a fresh-bread odor and reduced the yeasty, doughy odor of the control roll but slightly increased its sweetness. On the other hand, musty overtones were detected by some, and this fact probably is responsible, in part, for lack of preference for the treated roll. The ferric chloride test on an alcoholic extract of the

TABLE III

FLAVOR TESTS ON YEAST ROLLS: MALTOL VS. CONTROL

FLAVOR DESCRIPTIONS (36 Tasters)	CONTROL	MALTOL, 0.1%
Odor		
Fresh bread	13	18
Yeasty, doughy, sour	16	11
Stale, musty, moldy	6	4
Sweet	0	5
Taste		
Fresh bread	15	21
Sweet	5	5
Sour, fermented	7	2
Stale, musty	3	0
Flat, bland	3	4
Bitter	3	3
Preference (No preference: 2)	12	22
Reason: More fresh-bread flavor	2	9
Sweeter, less off-flavor	9	14

baked roll was judged *weak*, hence little isomaltol remained to give flavor.

Maltol in yeast rolls give a decidedly fresher, more pleasing flavor according to the panel (Table III), than the control rolls which they judged sour, stale, and musty in comparison. The enol test was *strong*, showing that much of the maltol remained in the roll.

TABLE IV

FLAVOR TESTS ON YEAST ROLLS: ISOMALTOL VS. MALTOL.

FLAVOR DESCRIPTIONS	16 TASTERS		17 TASTERS	
	Isomaltol, 0.1%	Maltol, 0.1%	Isomaltol, 0.2%	Maltol, 0.2%
Odor				
Fresh or normal bread	7	7	7	12
Yeasty	5	4	1	6
Sweet	3	1	1	2
Sour, sharp	1	2	2	0
Stale, musty	2	1	4	0
Crackers	1	0	0	0
Taste				
Fresh or normal bread	9	6	10	11
Yeasty	2	2	0	3
Sweet	1	3	5	4
Stale, musty	3	1	3	0
Flat, bland	1	3	1	3
Fatty, buttery	1	0	1	1
Nutty	0	0	2	0
Melony, cucumber	1	0	1	0
Preference[a]	6	9	5	11
Reason: More flavor	3	4	2	1
Sweeter, less off-flavor	2	3	3	10
Very little difference	1	2	1	0

[a] No preference expressed by one taster at each session.

In flavor comparisons of isomaltol against maltol (Table IV), the maltol-treated rolls were preferred. The results show again that isomaltol diminishes yeasty odor and taste, but accompanying mustiness reduces acceptability. Ferric chloride tests on the finished rolls again showed *weak* enolic content for isomaltol-treated rolls and *strong* enolic content for maltol-treated rolls. Evidently isomaltol steam-distills to a much greater extent than maltol during baking. When the initial isomaltol concentration was doubled to 0.2%, the enol content was still much less than the enol content of the 0.1% maltol-treated roll by the ferric chloride test. The greater preference for maltol, therefore, can be attributed to its higher concentration in the finished bread and to its being free from musty, stale off-flavors.

Because *O*-galactosylisomaltol is a beta-galactoside readily split by almond emulsin to galactose and isomaltol (5), tests were made to

determine whether an active beta-galactosidase is contained in yeast rolls. Dough containing 0.3% *O*-galactosylisomaltol was allowed to rise normally. Ferric choloride tests on the risen dough showed purple color and *medium* enol content. Therefore, flavor evaluations were made on such treated rolls baked, as before, at 218°C. Table V shows that preference was slightly less for these rolls than for the control

TABLE V

O-GALACTOSYLISOMALTOL IN WATER, YEAST ROLLS, AND PASTRY

MEDIUM	TEST No.	CONCENTRATION	TASTERS	FLAVOR RESPONSE	
		%	*No.*		
Water					
Doubly distilled, pH 6	1	0.15	16	Tasteless	11
				Bitter	4
				Sour	1
	2	0.50	19	Tasteless	4
				Bitter	13
				Sour	2
				Preference over Control	
				%	
Yeast rolls					
Baked at 218°C[a]	1	0.30	16	43	
	2	0.50	19	47	
	3	0.50	14	46	
Pie crust					
Baked at 260°C[b]	1	0.50	14	69	
	2	0.50	18	83[c]	
	3	0.50	17	60	

[a] Enol test *medium* in dough, *weak* after baking.
[b] Enol test *weak* after baking.
[c] Significant at the 5% level.

bread. Table V also shows that the flavor of *O*-galactosylisomaltol at 0.5% concentration in water is decidedly bitter. However, bitterness was not detected in treated rolls; the evaluation was generally the same as for the controls, but with more "sweet" responses. In every case, the treated rolls browned more than the controls. The enol tests were *negative* (or very weakly positive), so it is concluded that the galactoside was split to galactose and isomaltol but that the isomaltol was almost completely distilled from the bread. The liberated galactose remained and gave rise to greater browning by caramelization and/or the Maillard reaction.

Tests on Pastry. Because *O*-galactosylisomaltol melts and decomposes with the sublimation of isomaltol around 200°C. (5), pie crust with a baking temperature of 260°C. was selected as a substrate for splitting the galactoside *in situ*. When water-recrystallized *O*-galactosylisomaltol in the low-melting crystalline form (m.p. about 190°C.) was used, ferric chloride tests showed *weak* to *medium* enolic contents in the finished

pie crust. Preferences were for the treated pie crust (Table V), although only one of three tests showed significance at the 5% level. Reasons for the preference were given by panel members as more baked flavor (biscuit, cracker) and less lardy, doughy, or rancid flavor. Whereas more baked flavor could have arisen by carmelization of the reducing sugar released, diminution in lardy and doughy flavor is the same response received for isomaltol alone in yeast rolls (Tables II and IV). More tests are needed to relate the preference to the galactose or isomaltol moieties individually, or to them both.

Results of all tests show that incorporation of maltol, isomaltol, and *O*-galactosylisomaltol in baked goods may significantly improve their acceptance. The concentrations of isomaltol and maltol in ordinary breads have not been determined. With isomaltol now available as a reference compound, and with this demonstration that isomaltol and maltol *can* be contributing components to fresh-bread odor and flavor, modern methods of analysis can be used to determine the importance of these compounds among the many volatile constituents that contribute to bread flavor.

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THE CARBOHYDRATES OF THE GRAMINEAE
XI. The Constitution of the Water-Soluble Polysaccharides Derived from Bread Crumb[1]

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ABSTRACT

The so-called "soluble starch" of bread consists of starch and a pentosan. "Soluble starch" from fresh bread represented, on a dry weight basis, 4.3% of the crumb and contained 11.7% pentosans; whereas, in stale bread from the same lot, the "soluble starch" represented 3.3% of the crumb and contained 19.3% pentosans. Water-soluble pentosans present in the "soluble starch" inhibited the retrogradation of amylose. Acetylation of the "soluble starch" fraction followed by fractional precipitation of the resulting acetate yielded a fraction, $[\alpha]_D^{27} - 44.5°$ in pyridine, which upon deacetylation and hydrolysis gave a polysaccharide complex containing xylose, arabinose, and glucose in the mole ratio five to four to three (5:4:3) respectively. This pentosan-rich fraction consumed 0.87 mole of periodate per pentose residue with the formation of formic acid corresponding to an average "repeating unit" of about 9.

The pentosan fraction was methylated via the acetate and fractionally precipitated. Hydrolysis and quantitative analysis of the resulting glycosides by the phenol-sulfuric acid procedure showed D-xylose (1 mol.), 2-*O*-methyl-D-xylose (1 mol.), 2,3-di-*O*-methyl-D-xylose (2.7 mol.), and 2,3,5-tri-*O*-methyl-L-arabinose (1.7 mol.). These methylation results show that the pentosan in the water-soluble polysaccharides of bread crumb possesses a highly branched structure and that it is structurally similar to the pentosan in the original flour.

It has long been known that a white amorphous powder, commonly called "soluble starch," can be isolated from bread crumb by extraction with cold water and precipitation with ethanol. The amount of "soluble starch" which can be obtained from bread crumb usually decreases upon storage (17) and, since "soluble starch" has a low iodine affinity, it has been postulated that the staling process involves association of amylopectin (26).

Unfortunately, it appears that to date only physical testing methods have been employed for the evaluation of "soluble starch" (8). The commonly employed iodine sorption (6) and fractional precipitation (7) techniques are not adequate for ascertaining the relative homoge-

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neity or heterogeneity of carbohydrate systems (14). Moreover, it appears that no chemical analysis of "soluble starch" has heretofore been undertaken.

The present paper is concerned with a study of the chemical composition and structure of "soluble starch" fractions obtained from fresh and stale bread.

Methods and Materials

Micro-Kjeldahl. The micro-Kjeldahl determination combined the digestion with selenium oxychloride (23) with the distillation and titration procedure of Ma and Zuazaga (18); perchloric acid was omitted from the digestion.

Pentosan. Total pentosans were determined by the procedure of Bailey (4).

Sugars. Sugar determinations were conducted by the phenol-sulfuric acid method (10) or the ferricyanide procedure (15).

Polysaccharide Composition. A 20-mg. sample was sufficient for hydrolysis, deionization, and chromatographic analysis as described by Gilles and Smith (14).

Acetyl Value. The acetyl value of acetylated polysaccharides was determined by the alcoholic alkali method (11).

Methoxyl Value. The methoxyl value of methylated polysaccharides was determined by the Zeisel volumetric method (5).

Methylation. Initial methoxyl groups were introduced by the modification of the Haworth procedure as applied to cereal pentosans (12,19,20). To effect complete methylation the Purdie method (25) was applied to partially methylated products which had been treated by the Haworth procedure.

Acetylation. The wheat gum was dispersed in pyridine and acetic anhydride by vigorously agitating the reactants in a Waring Blendor. After standing at room temperature until the heat of reaction had been dissipated, the solution was poured into ice water and the precipitated acetate was collected by filtration, washed with water to remove pyridine and acetic acid, and dried by suction.

Fractional Precipitation. Petroleum ether (b.p. 30°–60°C.) was added with stirring to a solution of the pentosan acetates in the least amount of acetone.

Deacetylation. The acetylated wheat gum (1 g.) was dissolved in acetone (25 ml.) and the solution was refluxed for a few minutes in the presence of aqueous 15% sodium hydroxide solution (20 ml.). The solution was cooled, acidified with glacial acetic acid, and poured into methanol. The deacetylated product was purified by reprecipitation

from aqueous solution with ethanol in the usual manner (yield, 0.5 g.).

Ash. The "straight" ashing method was used (3).

Periodate Oxidation. The method of Abdel-Akher and Smith (1) was used for periodate oxidation studies on the deacetylated pentosans.

Micropipets. Micropipets were used to apply the sugar solutions to all chromatograms used for quantitative analysis. These pipets were made from small-bore tubing, the tips being ground flat with carborundum dust on a glass plate; the pipets were standardized to deliver with constant-boiling hydrochloric acid.

Phenol Reagents. Reagent grade phenol was distilled in a glass apparatus, and stored in a dark Pyrex bottle in the dark. Suitable dilutions were prepared with distilled water as needed.

Chromatographic Sprays. For general detection of the reducing sugars, a spray of ammoniacal silver nitrate (21) or *p*-anisidine (16) was used. For methylated sugars, sprays of *p*-anisidine or N,N-dimethyl-*p*-aminoaniline (16) were used.

Water-Solubles from Wheat Flour. Water-solubles were prepared by extracting unbleached and unbromated hard wheat flour (1 kg. Southwest Bakers' Patent) with two portions (2.5 liters each) of distilled water at 5°C. under a nitrogen atmosphere. The combined extracts were centrifuged and dried from the frozen state. This water-soluble material (16.7 g.) was dissolved in 250 ml. of distilled water and the solution was dialyzed at 5°C. against five changes of water for 5 days. The solution containing the nondialyzable material was centrifuged (1.9 g. of insoluble residue) and the supernatant made up to 300 ml. (solution A). An aliquot (50 ml.) of solution A when treated with ethyl alcohol gave a precipitate which when washed and dried amounted to 1.0 g.

"Soluble Starch" from Fresh and Stale Bread. The "soluble starch" was extracted from bread crumb (1 kg.) with two portions (3 and 2 liters, respectively) of distilled water at 30°C. The combined extracts were centrifuged, concentrated under reduced pressure to about 9 liters, treated with absolute ethanol (38 liters), digested 1 hour in a steam bath, and allowed to stand overnight. The precipitated product was filtered, washed with absolute ethanol, and dried *in vacuo*.

Samples of "soluble starch" were prepared from "stale" and "fresh" bread. The "stale" sample was derived from a commercial white crumb which had been baked without the aid of emulsifying agents and had been stored for 5 days at 5°C. The "fresh" sample was extracted 2 hours after baking from the same batch of commercial bread (7). Both samples of "soluble starch" were white amorphous powders, sparingly soluble in water, giving a positive pentosan test (pink color) when

boiled with 12% hydrochloric acid solution and tested with aniline acetate paper; they also gave a blue color with iodine.

Results

Chemical Composition. General chemical tests were applied to characterize the "soluble starches." The properties and composition of the so-called "soluble starch" from fresh and stale bread are recorded in Table I.

TABLE I

ANALYTICAL DATA DESCRIBING THE "SOLUBLE STARCH" EXTRACTED FROM "FRESH" AND "STALE" BREAD CRUMB

ANALYTICAL TEST	"SOLUBLE STARCH"	
	"Fresh" Bread	"Stale" Bread
$[\alpha]_D^{25}$ *N* NaOH (c, 1)	+87.2°	+24.3°
Pentosan (%)	11.7	19.3
Nitrogen, mg/g	8.78	12.51
Ash (%)	4.94	7.20
Sugar components (by chromatography)	Glucose, arabinose, xylose	Glucose, arabinose, xylose
Percent of total crumb (dry wt. basis)	4.33	3.27

Both "fresh" and "stale" samples of "soluble starch" gave upon hydrolysis D-glucose, L-arabinose, and D-xylose. A significant point of difference in these "soluble starches" was observed in the specific rotation of their aqueous solutions. The "soluble starch" from "fresh" bread had $[\alpha]_D^{25} + 87.2$, whereas that from "stale" bread had $[\alpha]_D^{25} + 24.3$. Moreover, the "soluble starch" from "fresh" bread contained less pentosan (11.7%) than did the "soluble starch" from "stale" bread crumb (19.3%).

Samples of "soluble starch" were hydrolyzed and chromatogramed and the component sugars determined by the phenol-sulfuric acid procedure (10). The hydrolyzed "soluble starch" fraction isolated from "fresh" bread contained D-glucose, L-arabinose, and D-xylose in the mole ratio of 5:1:1, whereas, in the same bread which had been subjected to 5 days' storage at 5°C., the mole ratios of the same three sugars were 3.3:2:1 respectively.

A similar change in the relative amounts of these monosaccharides was observed in samples of "soluble starch" extracted from bread baked with emulsifying agents which were furnished by the American Institute of Baking. The D-glucose, L-arabinose, and D-xylose mole ratio in the bread 2 hours old was 2.5:0.8:1, whereas the corresponding mole ratio in the "stale" bread sample (96 hours old) was 1.88:0.75:1.

The ratio of D-glucose to pentose in the "soluble starch" decreases during the staling process. This change apparently occurs regardless of the presence or absence of emulsifying agents and may indicate that amylose or amylopectin, or both, spontaneously retrograde as staling progresses.

Acetylation of "Soluble Starch." A sample of "soluble starch" extracted from "stale" bread, which had been stored 5 days at 5°C., was acetylated in the Waring Blendor. The acetate was isolated as a stringy mass by pouring into water and was freed from impurities by washing with water, methanol, and finally with ether. The crude product was dissolved in acetone and subjected to fractional precipitation by addition of increasing amounts of petroleum ether. A summary of these results is given in Table II.

TABLE II

FRACTIONATION OF THE ACETATE OF THE POLYSACCHARIDE FROM "STALE" BREAD

FRACTION	PET. ETHER[a] ADDED TO ACETONE SOLUTION (600 ML.)	WEIGHT	$[\alpha]_D^{23}$ (ACETONE) (c, 0.5)
	ml	*g*	
I	300	7.60	−5.5°[b]
II	90	0.15	+106°
III	150	3.00	+135°
IV	100	3.50	+133°
V	150	3.40	+143°
VI	200	1.85	+157°
VII (by evaporation of mother liquors)		0.40	+157°[c]

[a] B.p. 30°-60°C.
[b] $[\alpha]_D^{23}$ in pyridine.
[c] $[\alpha]_D^{23}$ + 146° in pyridine.

Repetition of the experiment confirmed these findings. Fraction I (combined material from two experiments) was dissolved in pyridine (200 ml.) and, after addition of acetone (75 ml.), fractionation was effected by addition of diethyl ether followed by petroleum ether in quantities shown in Table III.

This fractionation appeared to furnish two components, one pos-

TABLE III

REFRACTIONATION OF THE PENTOSAN ACETATE FRACTION I FROM "STALE" BREAD

FRACTION	SOLVENT ADDED SUCCESSIVELY TO ACETONE-PYRIDINE SOLUTION[a]	WEIGHT	$[\alpha]_D^{27}$ PYRIDINE (c, 1.0)
		g	
A	600 ml. diethyl ether	4.45	−28°
B	25 ml. pet. ether	5.30	−44.5°
C	50 ml. pet. ether	1.00	+123°
D (by evaporation of mother liquors		2.20	+104°

[a] Acetone, 50 ml.; pyridine, 200 ml.

sessing a negative specific optical rotation, the other a positive rotation. Inasmuch as a study of barley gum (13) had shown that the pentosan components were concentrated in fractions with a negative specific rotation, fraction B was chosen for further work on the structure of the pentosans of "soluble starch."

Examination of Fraction B of the Acetate of the Pentosan Fraction. A sample of fraction B of the acetate was deacetylated and purified by reprecipitation. The deacetylated fraction B was hydrolyzed with *N* sulfuric acid and subjected to chromatographic analysis in the usual manner. By means of the phenol-sulfuric method, it was found that the molar ratio of the component sugars glucose:arabinose:xylose was 0.6:0.8:1.0.

In periodate oxidation studies, 0.87 mol. of periodate was consumed per "pentose" unit with the liberation of 1 mol. of formic acid per 8.8 mol. of pentose.

Methylation of "Soluble Starch" from "Stale" Bread. (a) *Haworth method.* A portion (4 g.) of the acetylated "soluble starch" fraction B was dissolved in a mixture of acetone (50 ml.) and 1,4-dioxan (125 ml.). The solution was subjected to the Haworth methylation procedure and the product collected by centrifugation.

After the second methylation the product was recovered by filtering through linen on a Büchner funnel and washing with boiling water. As the methylation proceeded, the solubility of the product in acetone increased. In all, six methylations were applied.

To obtain the greatest possible yield of the methylated pentosan, the supernatants from the first and second methylations were dialyzed against water to remove salts, concentrated by pervaporation, and subjected to four methylations by the same Haworth procedure. The methylated polysaccharide thus obtained was combined with the product from the sixth methylation of the original pentosan and the combined mixture was methylated once more.

The methylated pentosan was washed with boiling water and dissolved in chloroform, and the solution dried over anhydrous magnesium sulfate. Upon concentration of the chloroform solution, a friable, amber-colored product was isolated (yield 1.55 g., OCH_3, 36.0).

(b) *Purdie method.* A solution of the methylated pentosan (1.5 g.) in methyl iodide (15 ml.) was refluxed in the presence of silver oxide (5 g.) to effect complete methylation. The methylated product was recovered in the usual manner (25). In all, four Purdie methylations were applied. The methylated pentosan from "soluble starch," yield 1.2 g., was a friable, glasslike substance which showed $[\alpha]_D^{25} - 119.5°$ in acetone (c, 2.1). Found: OCH_3, 38.6. The methylated pentosan gave

a very faint mauve color with iodine.

Fractionation of Methylated Pentosan. The methylated pentosan (1.2 g.) was dissolved in acetone (65 ml.) and subjected to fractional precipitation with petroleum ether in the usual manner. The fractions derived from the primary fractionation were as follows:

Fraction	*Weight* mg.	$[\alpha]_D^{25}$ *Acetone*
1	306.6	−93.0
2	550.3	−154.5
3	171.2	−147.0
4	158.4	±0.0

Fractions 1, 2, and 3 were then refractionated in the same manner to give the following subfractions:

Fraction	*Weight* mg.	$[\alpha]_D^{25}$ *Acetone*	*Solvent Precipitation Data Ratio of Acetone: Pet. Ether*
1A	145.4	−66°	Insoluble in 1:0.7
1B	142.2	−122°	Soluble in 1:0.7
2A	14 (approx.)	...	Insoluble in 1:0.8
2B	535.4	−153°	Soluble in 1:0.8
3A	143.7	−150.5°	Insoluble in 1:2.0
3B	27.4	−67°	Soluble in 1:2.0

Fractions 2B and 3A were combined, giving a composite sample which showed $[\alpha]_D^{24} - 152.5°$ in acetone (c, 3.4) and had $-OCH_3$, 38.9. A marked similarity exists between this material and the methylated pentosan derived from barley gum, which showed $[\alpha]_D^{24} - 160°$ in acetone (12).

Analysis of the Methylated Pentosan. (a) *Hydrolysis.* The methylated pentosan (0.6154 g.) was refluxed for 15 hours with 20 parts of 2% methanolic hydrogen chloride solution, after which time the rotation had reached a constant value of $[\alpha]_D^{24} + 130°$. Hydrolysis of the mixed glycosides thus formed with *N* hydrochloric acid for 20 hours gave a hydrolysate with $[\alpha]_D^{24} + 13°$ (in the hydrolysis medium).

(b) *Chromatographic analysis.* (i) Qualitative. Partition chromatographic analysis of the neutralized hydrolysate using both 1-butanol:ethanol:water and butanone:water azeotrope revealed the presence of D-xylose, 2-*O*-methyl-D-xylose, 2:3-di-*O*-methyl-D-xylose, and 2:3:5-tri-*O*-methyl-L-arabinose, the R_f values of which are given in Table IV.

(ii) Quantitative. The analysis of the components separated on chromatograms irrigated either with 1-butanol:ethanol:water or with butanone:water azeotrope determined by the phenol-sulfuric acid procedure (10) in the usual manner was as follows: D-xylose (1 mol.), 2-*O*-methyl-D-xylose (1 mol.), 2:3-di-*O*-methyl-D-xylose (2.7 mols.), and 2:3:5-tri-*O*-methyl-L-arabinose (1.7 mols.).

Preparation of Derivatives. (a) *Isolation of components by chromatography.* The hydrolysate was placed on a sheet of Whatman No. 3

TABLE IV

PARTITION CHROMATOGRAPHIC ANALYSIS OF THE HYDROLYSATE OF METHYLATED BREAD PENTOSAN

Component	R_f Value	
	Butanone:Water	1-Butanol:Ethanol:Water
1. D-xylose	0.06	0.20
2. 2-*O*-Methyl-D-xylose	0.20	0.40
3. 2,3-di-*O*-Methyl-D-xylose	0.60	0.64
4. 2,3,5-tri-*O*-Methyl-L-arabinose	0.89	0.79

filter paper in two lines each 13.5 cm. in length, 8 cm. from the edge, and 7 cm. from the top of the wide edge (57 cm.) of the paper. Control strips were run on the outer margins (4 cm.) and at the center of the paper (6 cm.). This procedure allowed for two blank strips (4 cm.) adjacent to each strip containing the hydrolysate; the chromatograms were developed in the usual manner and the sugar components eluted from the appropriate areas.

The solution containing each sugar derivative was filtered through sintered glass, concentrated under reduced pressure, dried, and weighed. These data, which appear in Table V, confirm the results of the colorimetric analysis. Although they do not agree exactly, it must be remembered that in the phenol-sulfuric acid procedure a correction is made for the contaminant eluted from the paper, whereas these data represent direct weights of the entire eluted material, sugar, and contaminant.

(b) *Identification of the components in the hydrolysate of the methylated araboxylan.* (1) D-Xylose. The component corresponding to D-xylose ($[\alpha]_D^{27} + 17.4°$ in water) crystallized upon nucleation. After recrystallization from methanol it had m.p. and mixed m.p. 145°C.

TABLE V

THE CLEAVAGE PRODUCTS OF THE METHYLATED ARABO-XYLAN DERIVED FROM THE WATER-SOLUBLE COMPONENT OF BREAD CRUMB

Sugar Derivative	Amount		$[\alpha]_D^{27}$ (Solvent)	Structural Feature Indicated
	Weight	Moles		
	mg			
D-xylose	41.6	1.0	+17.5 (water)	→ 4 − D − xyl *p* 1 → (3 ↑ 2 ↑)
2-*O*-Methyl-D-xylose	49.0	1.0	+22.0 (methanol) +16.5 (ethanol)	→ 4 D − xyl *p* 1 → (3 ↑)
2,3-di-*O*-Methyl-D-xylose	153.9	3.1	+19.0 (methanol)	→ 4 D − xyl *p* 1 →
2,3,5-tri-*O*-Methyl-L-arabinose	67.0	1.3	−24.0 (water) −32.0 (methanol)	L − ara *f* 1 →

Treatment of the D-xylose (21 mg.) with a solution of benzaldehyde in methanolic hydrogen chloride for 1 day at room temperature and for 2 hours at 3°C. gave di-*O*-benzylidine-D-xylose dimethylacetal, m.p. and mixed m.p. 208°, $[\alpha]_D^{21.5} - 10.3°$ in chloroform (c, 0.6). An authentic specimen (9) had m.p. 207°C., $[\alpha]_D^{20} - 9°$ in chloroform.

(2) 2-*O*-Methyl-D-xylose. This component, showing $[\alpha]_D^{27} + 21.7°$ in methanol and + 16.3° in ethanol, crystallized upon nucleation with an authentic specimen of 2-*O*-methyl-D-xylose, and after recrystallization from ethanol it had m.p. and mixed m.p. 132°C.

(3) 2,3-Di-*O*-methyl-D-xylose. This fraction, which corresponded chromatographically to 2,3-di-*O*-methyl-D-xylose, had $[\alpha]_D^{27} + 18.7°$ in methanol. A solution of the 2,3-di-*O*-methyl-D-xylose (130 mg.) in ethanol (3 ml.) containing aniline (0.2 ml.) was refluxed for 3 hours. Upon removal of solvent the product crystallized spontaneously. After successive washings with ethylacetate and a mixture of petroleum ether, ethanol, and ethylacetate, the *N*-phenyl-2,3-di-*O*-methyl-D-xylosylamine had m.p. and mixed m.p. 131.5°C., $[\alpha]_D^{21.5} + 182°$ in ethyl acetate (c, 0.5). An authentic specimen had m.p. 132°C., and $[\alpha]_D^{22}$ + 181° in ethyl acetate (c, 0.8).

(4) 2:3:5-Tri-*O*-Methyl-L-Arabinose. The trimethyl-L-arabinose fraction (46 mg.) having $[\alpha]_D^{27} - 31.9°$ in methanol was dissolved in water (10 ml.) and bromine was added. The solution was kept in a dark cabinet at room temperature (26°C.) for 88 hours. Bromine was removed by aeration and the bromide ions removed by addition of silver oxide. The mixture was filtered, passed through a cation exchange resin ("Amberlite" IR 120 H form), and concentrated under reduced pressure. Methanol (3 ml.) was added and the methanolic solution saturated with gaseous ammonia. The solvent was removed under reduced pressure and the syrup was nucleated with 2:3:5-tri-*O*-methyl-L-arabonamide. When kept at 3°C. the characteristic needlelike crystals of the amide appeared, but not enough crystals were isolated to effect characterization.

The 2:3:5-tri-*O*-methyl-L-arabonamide was hydrolyzed with barium hydroxide solution, and the solution passed through a cation exchange resin, "Amberlite" IR 120, concentrated under reduced pressure, diluted with 5 drops of water, and chromatogramed with 1-butanol: ethanol:water. The chromatogram, sprayed with an alcoholic hydroxylamine spray (2), revealed the presence of 2:3:5-tri-*O*-methyl-L-arabonolactone (R_f 0.84). An authentic specimen of 2:3:5-tri-*O*-methyl-L-arabonamide treated in the above manner gave the same results.

Additional evidence for the presence of 2:3:5-tri-*O*-methyl-L-arabinose arises from the comparison of the spectral absorption curves of

the color produced by the phenol-sulfuric acid reaction with methylated sugar derivatives having R_f values similar to that possessed by 2:3:5-tri-*O*-methyl-L-arabinose. Authentic 2:3:5-tri-*O*-methyl-L-arabinose and its glycoside displayed a maximum spectral absorption of 415 mμ; the unknown material isolated from the hydrolysate of the methylated bread pentosan, which possessed R_f 0.87, displayed a maximum spectral absorption at 415 mμ and no secondary peak, whereas authentic 2:3:4-tri-*O*-methyl-D-xylose and 2:3:6-tetra-*O*-methyl-D-glucose displayed maximum absorption peaks at 485 and 490 mμ, respectively.

Effect of Water-Solubles on Amylose Retrogradation. The water-soluble components of hard wheat flour were dialyzed to remove the low-molecular-weight components. The nondialyzable material was made up to volume (solution A). Aliquots were added to dilute aqueous solutions of amylose. The solutions were stored at 5°C. and the transmittances were read periodically at 420 mμ in an Evelyn colorimeter against water as a blank. Typical data follow:

Test Solution	*Percent Transmittance*			
(5 ml. of each solution were used)	*0 hours*	*17.5 hours*	*24 hours*	*114 hours*
0.4% amylose + water	97	ppt	..	..
0.4% amylose + solution A (water-solubles)	71	71	71	72
Water + solution A (water-solubles)	78	77	77	77

The data indicated that amylose precipitated spontaneously from a dilute aqueous solution. However, in the presence of the water-solubles, retrogradation of amylose was inhibited.

Discussion

The water-soluble polysaccharides, commonly called "soluble starch," which may be derived from bread crumb contain as monosaccharide building units primarily glucose, arabinose, and xylose. The term "soluble starch" is therefore a misnomer. In the so-called "soluble starch" fraction of "fresh" bread, glucose, arabinose, and xylose were found in the mole ratio of 5:1:1, whereas in the same bread which had been kept for 5 days at 5°C. the mole ratio of the three sugars was 3.3:2:1 respectively. These results show that the relative amount of pentose-containing polysaccharides in the "soluble starch" increases as bread stales and the glucose-containing component decreases. This is a consequence of the retrogradation of amylose and/or association of amylopectin as staling progresses (17,26).

These results are interesting, particularly when considered in the

light of the following facts. The total water-solubles of wheat flour, after dialysis to remove any low-molecular-weight compounds, were found to inhibit the retrogradation of a 0.2% aqueous solution of amylose at 5°C. for approximately 5 days; the control showed nearly complete retrogradation in 18 hours. Also, the water-solubles, although affecting the properties of baked bread (22), do not affect the staling rate. The "squeegee" fraction of wheat flour (19) also is said to have no effect on the staling rate. Inasmuch as the proportion of pentosan in the soluble starch from "fresh" bread is 0.51% (4.33 × 11.7) and from stale bread, 0.63% (3.27 × 19.3), it would seem that the pentosan-rich fractions of flour are not primarily involved in the staling process (see 19,20,24). Since these materials retard the retrogradation of amylose, it appears that the decrease in percentage of soluble starch which occurs during staling is mainly due to a change of state in the amylopectin. This work lends support, therefore, to the previous suggestion (26) that the amylopectin component of starch undergoes aggregation during the staling of bread.

The pentosan of "soluble starch," composed of D-xylose and L-arabinose, may be described as an arabo-xylan. From the isolation of the 2,3-di-*O*- and 2-*O*-methyl derivatives of D-xylose, it is evident that the polysaccharide is structurally related to other plant xylans and that the linear portions of the molecule are joined by 1→4 bonds. Branching, which is quite extensive since the ratio of 2-*O*-methyl-D-xylose and D-xylose to the 2,3-di-*O*-methyl derivative is 2:2.7, takes place through C_3 and through C_2 and C_3 of a relatively large proportion of the 1→4-linked D-xylose residues (see Table V). The isolation of the L-arabinose as the 2,3,5-tri-*O*-methyl derivative shows that the L-arabinose units are present in the furanose form. In addition, the isolation of only the 2,3,5-tri-*O*-methyl derivative of L-arabinose proves that all of the arabofuranose units constitute the terminal nonreducing ends in the pentosan molecule. The high negative rotation of the araboxylan of "soluble starch" shows that most of the 1→4 linkages between the D-xylopyranose residues are of the beta type, and for the same reason the L-arabofuranose units are most probably of the alpha type.

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MODIFIED WOHLGEMUTH METHODS FOR ALPHA-AMYLASE ACTIVITY OF WHEAT AND RYE[1]

SVEN HAGBERG[2]

ABSTRACT

Modified methods for determining alpha-amylase activity of wheat and rye grain or flour based on the Wohlgemuth principle are described. These can be carried out comparatively rapidly and give values in SKB units. The end-point is determined by using either a permanent color standard or by taking colorimetric readings at fixed time intervals.

Among methods for determining alpha-amylase activity based on the Wohlgemuth principle, the method of Sandstedt, Kneen, and Blish (16) as described in *Cereal Laboratory Methods* (1) has achieved widespread popularity. This method was originally intended for malt, but modifications for wheat and rye have been suggested by Kneen and Sandstedt (8), Kneen, Sandstedt, and Hollenbeck (9), Olson, Evans, and Dickson (13), Redfern (14), and Stone (17). In Germany and some other countries the methods of Ritter (15) and Lemmerzahl (11) are used. These modified Wohlgemuth methods are either time-consuming and difficult to carry out, or the relation between the values obtained and the well-known SKB units have not been determined.

Hanes and Cattle (4) were the first to show that the measurement of absorbancy could be used to follow quantitatively the alteration in the iodine coloring property of starch during its degradation by amylases. Bawden and Artis (2) have reported colorimetric methods for evaluating alpha-amylase activity of malt. Similar methods for wheat and rye have been used by Hoskam (5), Jongh (6), Knight (10), Olerod (12), and Hagberg (3).

The object of the present study was to develop rapid, accurate methods based on the Wohlgemuth principle for determination of alpha-amylase activity of wheat and rye and its expression in SKB units.

Materials

Two samples each of wheat, wheat flour, rye, and rye flour were used in this study. Each pair of samples was blended to give three additional samples with activities ranging between those of the unmixed samples (see Table I).

The wheat and rye samples were finely pulverized according to

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TABLE I

ANALYTICAL DATA FOR WHEAT, RYE, AND FLOUR SAMPLES

MATERIAL	SAMPLE NO.	ASH	PROTEIN	ALPHA-AMYLASE ACTIVITY
		%	%	*SKB units*
Wheat flour	1	0.52	12.3	0.06
Wheat flour	5	0.66	9.6	6.00
Rye flour	6	0.77	8.7	0.13
Rye flour	10	0.68	7.0	8.10
Ground whole wheat	11	..	..	0.08
Ground whole wheat	15	..	..	1.53
Ground whole rye	16	..	..	0.05
Ground whole rye	20	..	..	4.34

Cereal Laboratory Methods (1). Ninety percent of the ground material passed through a No. 30 standard sieve.

I. A Modified AACC Method for Determination of Alpha-Amylase

This method is performed similarly to that described in *Cereal Laboratory Methods* (1) but using the glass disk No. 620-S5 in a Hellige Comparator No. 607 or comparable device as suggested by Redfern (14). The Comparator should be illuminated by an ordinary 100-watt frosted bulb mounted 6 in. from the rear opal glass of the Comparator, in such a manner that direct rays from the lamp do not shine in the operator's eyes. Laboratories which do not have the Hellige equipment may use a stable colored solution giving the same results as the Hellige disk. The solution suggested by Landis and modified by Olson, Evans, and Dickson (13) gives a more reddish color than the Hellige disk, when an ordinary 100-watt frosted bulb is used. However, the following solution gives practically the same color as disk No. 620-S5 when compared in the Hellige Comparator: 19.250 g. cobaltous chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 2.567 g. potassium dichromate, and 2.565 g. cupric sulfate pentahydrate per 100 ml. 0.01*N* hydrochloric acid solution.

Apparatus and Reagents. The apparatus and reagents are the same as those described in *Cereal Laboratory Methods* (1), with the following exceptions:

(1) Dilute iodine solution. Dissolve 30 g. potassium iodide in water, add 3 ml. stock iodine solution, and make up to 500 ml. This solution should be at, or close to, 30°C. when color comparisons are made. Make fresh solution daily.

(2) Calcium chloride solution. Use 0.2% solution for all extracts.

(3) Buffered beta-amylase limit dextrin solution. This may be the same as described in *Cereal Laboratory Methods* (1) or it may be a buffered solution containing an equivalent amount of beta-amylase limit dextrin (erythrodextrin) in the solid state prepared as described by Hoskam (5) and Jongh

(6).[3] One gram of "starch" as understood in the AACC method (1) is equivalent to about 0.4 g. of erythrodextrin on a dry-weight basis.

Preparation of Extract. Extract 5 g. of flour or finely pulverized grain with 100 ml. of 0.2% calcium chloride solution for 1 hour at 30°C., mixing by rotation every 15 minutes. Centrifuge at 2,500 r.p.m. (Corda centrifuge for 100-ml. tubes, mean radius 10 cm.) for 10 minutes and filter the supernatant liquor through No. 42 Whatman filter paper. Refilter the first part of the filtrate until a clear filtrate is obtained. Dilute the extract suitably with 0.2% calcium chloride solution. Bring this extract to 30°C. before making the activity determination.

Determination of Alpha-Amylase Activity. Transfer 30 ml. of the extract to a 150-ml. test tube (about 32 by 200 mm.) or 125-ml. Erlenmeyer flask, and place in the 30°C. bath. After attempering to 30°C. add 10 ml. of the buffered beta-amylase limit dextrin solution and mix well. The relation between the volumes of extract (diluted if needed) and the dextrin solution should always be 3 to 1.

A. Ordinary Procedure for End-Point Determination (Modified AACC Method IA).

A series of test tubes (13 by 100-mm.) containing 5 ml. dilute iodine solution are prepared and attempered at 30°C. in readiness for testing. At appropriate intervals, 4 ml. of the hydrolyzing mixture are added from a fast-flowing pipet to the 5 ml. of iodine solution. It is important in this and all other similar methods that the pipet is not polluted with iodine during draining. This can be avoided by draining the pipet on the upper part of the test tube in which the iodine solution previously has been carefully conveyed by aid of a pipet into the lower part of the test tube. After mixing, the hydrolysate-iodine solution is poured into the 13-mm.-square tube for color comparison in the Hellige Comparator. After the color comparison is made, the solution is poured out by giving the tube a quick shake. In this way very little liquid remains in the tube and it is ready for another test. When the end-point is near, readings are made every 0.5-minute if the hydrolyzing time is 10 to 30 minutes, and the dextrinizing time is interpolated as the 0.25-minute between two readings (14).

For convenience it is desirable that the dextrinization times should fall between 10 and 30 minutes, but up to 120 minutes or more can be used. When the hydrolyzing time is more than 30 minutes, the interval between the readings can be extended in proportion to the prolonged hydrolyzing time.

Calculation. Calculate and express results in SKB units as indicated in *Cereal Laboratory Methods* (1). Typical results are shown in Table II.

B. Rapid Procedure for End-Point Determination (Modified AACC Method IB)

To decrease the hydrolyzing time required and to reduce the risk of the bluish discoloration mentioned later, in the "Discussion," modify the mixture for end-point determination as follows: mix 3 ml. of the hydrolyzing mixture with 10 ml. of an iodine solution containing 43 g. potassium iodide and 4.3 ml. stock iodine per 1,000 ml. Determine the end-point as described in Method IA. Calculate as indicated in *Cereal Laboratory Methods* (1) and multiply by 0.7 (empirically found factor) to express the values in SKB units. Typical results obtained by this method are given in Table II.

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[3] The author has corresponded with manufacturers for the purpose of having erythrodextrin in a solid state available in the market.

II. Colorimetric Method

Apparatus. (1) One water bath regulated at 30°C. and one at 20°C.[4]
(2) An instrument[5] capable of measuring absorbancy at 575 mμ.

Reagents. The reagents are the same as used in the Modified AACC Method IA, with the following exceptions:

(1) Dilute iodine solution. Dissolve 43 g. potassium iodide in water, add 4.3 ml. stock iodine solution, and make up to 1,000 ml. This solution should be at 20° or 30°C. when color comparisons are made. Make fresh solution daily.

(2) Blank for zero-point (BO). Mix 2 ml. of 0.2% calcium chloride solution, 10 ml. dilute iodine solution, and 40 ml. distilled water.

(3) Blank for starting-point (BS). Mix 10 ml. of dilute iodine solution, 40 ml. of distilled water, and 2 ml. of a mixture consisting of 1 part buffered beta-amylase limit dextrin solution and 3 parts of 0.2% calcium chloride solution. The BO and BS blanks described here have approximately the same absorbancies as corresponding mixtures containing flour extract. If individual BS blanks are prepared with each flour, the flour extract must be mixed with iodine solution before the limit dextrin is added.

Preparation of Extract and Hydrolyzing Mixture. Same as for Modified AACC Method IA.

Determination of "Period of Half-Life."[6] After mixing and attempering the BO and BS solutions exactly to 20° or 30°C., adjust the colorimeter so that the BO solution gives an absorbancy of 0 at 575 mμ, then make an absorbancy reading of the BS solution. After three or more appropriate hydrolyzing times (e.g. 10, 20, and 30 minutes), add from a fast-flowing pipet 2 ml. of the hydrolyzing mixture to solutions of 10 ml. dilute iodine solution and 40 ml. distilled water attempered exactly to 20°C., in a water bath; mix well and pour aliquots of the hydrolysate-iodine solutions into colorimeter tubes, and measure the absorbancy at 575 mμ at room temperature (about 20°C.). This makes it possible to start hydrolyzing several samples at intervals of 1 or 2 minutes. Five or ten samples can be tested in the same series. For flour with very low amylase activity it may be advisable to use more extended hydrolyzing times — e.g., 20, 40, and 60 minutes.

To prevent errors in absorbancy determinations it is important that the mixture is at 20°C., that the photometer cells (tubes) are clean, and that the readings are made immediately or preferably 10 minutes after the first mixing. Repeated mixing must also be made immediately before the absorbancy reading, and the reading should be made immediately after the cell has been inserted in the photometer.

Calculation of Alpha-Amylase Activity. For accuracy the concentration of the flour extract should be so adjusted that the absorbancy values used for calculation correspond to a "starch" conversion of 35 to 65%. For convenience and accuracy the hydrolyzing time should fall between 10 and 40 minutes (preferably 20 minutes). With a flour of low alpha-amylase activity 120 minutes or more can be used.

The absorbancy reading for BS (E_0 = the absorbancy after 0 minutes) and the readings after three different times (E_t = the absorbancy after t minutes) should fall on a straight line when plotted on semilogarithmic pa-

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[4] The temperature of most European laboratories is about 20°C.; that of American laboratories is about 30°C. The bath temperature selected for attempering the color-producing reagents will depend upon the laboratory temperature.

[5] A suitable inexpensive instrument is the "EEL" Colorimeter, Model A, Evans Electro-Selenium Limited, Harlow, England, using yellow filter No. 626, and standardized large tubes ⅝ in. in diameter (16 mm.), capacity 8 ml. The Spectronic "20" Colorimeter, Bausch & Lomb, using standardized ½ by 4-in. tubes, should give similar results but it has not been tested by the author.

[6] The time required for one-half of the erythrodextrin to be digested by the alpha-amylase.

per, whereupon the "period of half-life" is evaluated from the intersection of this line and a line (horizontal) for half of the absorbancy for BS ($=\frac{E_o}{2}$).

The period of half-life ($t_{\frac{1}{2}}$) in minutes can also be calculated from the formula:

$$t_{\frac{1}{2}} = t\,\frac{\log E_o - \log\frac{E_o}{2}}{\log E_o - \log E_t} = t\,\frac{0.30103}{\log E_o - \log E_t}$$

For accuracy calculate $t_{\frac{1}{2}}$ as the mean value based on two or preferably three readings following the equation for a first-order reaction.

Calculate the H unit as indicated in *Cereal Laboratory Methods* (1), using $t_{\frac{1}{2}}$ instead of "dextrinization time."

Tests performed have shown (Figs. 3 and 4) that:

One H unit × 0.42 = one SKB unit.

III. Rapid Colorimetric Method

This method, which is suitable for routine work, is similar to Method II, with the following changes:

Preparation of Extract. The extraction is limited to 5 minutes in a Waring Blendor (14,000 r.p.m.) with a temperature in the mixture after stirring of about 45°C.

Calculation of Alpha-Amylase Activity. Appropriate substitutions of data are made in the following equation for a first-order reaction:

$$V_1 = \frac{\text{"starch" (g)} \times (\log E_o - \log E_t)}{\text{flour equivalent (g)} \times \text{time (minutes)}}$$

When the relation of amount of "starch" and flour in the hydrolyzing mixtures is constant, the formula is simplified accordingly.

Tests performed have shown (Figs. 3 and 4) that:

$V_1 \times 0.82$ = SKB units.

IV. Mixture-Value Method

When the amylase activity of a sample is very low it requires a comparatively long time to reach the end-point, especially with the AACC method (1). Therefore, it can be convenient to determine the activity of a mixture containing appropriate amounts of a flour (preferably extract) of a sample of known and comparatively high alpha-amylase, and the sample with low unknown activity. The AACC method or any of the methods described above can be used to determine alpha-amylase activity of such a mixture. From these data the activity of the unknown sample can be calculated.

Example:

A = alpha-amylase activity of sample (a) in SKB units;

0.5 = alpha-amylase activity of sample (b) in SKB units;

0.095 = alpha-amylase activity of a mixture of 90% of (a) and 10% of (b);

$$A \times 0.9 + 0.5 \times 0.1 = 0.095;$$

$$A = 0.05 \text{ SKB units}$$

Thus, the mixture requires only 52.5% of the time required for sample (a) to reach the end-point. This procedure is often useful in practice and is more rapidly carried out than the method of Kneen, Sandstedt, and Miller (7), which involves prehydrolysis (about 18 hours) of the extract of the sample of low unknown activity, followed by a second hydrolysis after the addition of a known amount of alpha-amylase.

Results and Discussion

Preparation of Extract: Stone (17) and Jongh (6) used salt-solutions to extract alpha-amylase from flour. Stone (17) used an extraction time of 15 minutes with intensified stirring and Olered (12) extracted 3 to 5 minutes in a "Turmix."[7] The author has tested rapid extraction procedures (5, 10, or 15 minutes) using different stirring or revolving devices, and has found that stirring 5 minutes in a Waring Blendor can be used for rapid tests. However, the 1-hour extraction period was used in all but routine tests.

The use of highly concentrated extracts should be avoided, since they may cause a bluish discoloration which interferes with the end-point determination, especially in Method IA, where a maximum of 4 g. of wheat flour and 3 g. of rye flour should be employed. The absorbancy readings can also be influenced if the degree of starch conversion is higher than 65%.

Hydrolyzing temperatures over 30°C. Alpha-amylase activity can be determined more rapidly at temperatures higher than 30°C. (Table

TABLE II

APPROXIMATE TIME REQUIRED FOR DETERMINING THE ALPHA-AMYLASE ACTIVITY OF FLOURS BY DIFFERENT METHODS, WHEN USING A FLOUR EXTRACT CONCENTRATION OF 5 G. TO 100 ML. OF SOLUTION

Alpha-Amylase Activity	Hydrolyzing Temperature 30°C.				Hydrolyzing Temperature 40°C. Colorimetric Method II
	AACC Method[a]	Modified AACC Method 1A	Modified AACC Method 1B	Colorimetric Method II	
SKB units	*minutes*[b]	*minutes*	*minutes*	*minutes*	*minutes*
0.05	960	160	114	67	34
0.10	480	80	57	33.5	17
0.50	96	16.0	11.4	6.7	3.4
1.00	48	8.0	5.7	3.4	1.7
2.00	24	4.0	2.8	1.7	0.8
4.00	12	2	1.4	0.9	0.4
8.0	6	1	0.7	0.4	0.2

[a] See reference 1.
[b] Time to reach "end point."

[7] A high-speed stirrer manufactured by AB Turmix, Sveavägen 13, Stockholm, Sweden.

Fig. 1. Influence of composition of iodine solution on absorbancy values after varying degrees of "starch" hydrolysis during 40 minutes:

Curve	Hydrolyzing Mixture		Mixture for Absorbancy Measurements			
	"Starch" Employed (Conc. = 2%)	*Flour Extract (Conc. = Varying)*	*Hydrolysate*	*Iodine Solution (Diluted)*	*Iodine/ "Starch"*	*Potassium Iodide/ "Starch"*
	ml	*ml*	*ml*	*ml*	*mg/mg*	*mg/mg*
1	20	40	2	50	0.066	30
2	20	40	2	100	0.66	300
3	20	40	2	100	0.30	0.60
4	20	40	2	100	0.50	1.00

II). However, in evaluating the "end-point" with the Hellige disk, the hydrolysate-iodine mixture should have a temperature of 30°C.

End-Point Determination. One way to decrease hydrolyzing time is to increase the flour/erythrodextrin ratio. In Modified AACC Method IA, the amount of "starch" originally present and the concentration of iodine in the mixture for determining the end-point is the same as in the AACC method (1). In Method IB the amount of "starch" originally present in the mixture for the end-point determination is lower than in the AACC method (1) but it is still high enough to make color determination possible. In both Methods II and III more than 1 ml. of the hydrolyzing mixture has been used for the color determination for the purpose of improving reproducibility.

Especially for flours with low alpha-amylase activity, the AACC method (1) requires extremely long hydrolyzing time compared with the other methods (Table II).

Temperature of Solutions at Time of Absorbancy Readings. The temperature of the solutions for absorbancy readings is of great importance. Tests have shown that the factors for transforming H units and V_1 values into SKB units change from 0.42 to 0.33 and from 0.82 to 0.64, respectively, when the temperature of the solutions is increased from 20° to 30°C. Since a common room temperature is about 20°C., this temperature was chosen for the absorbancy readings in this study.

Evaluation of Absorbancy Readings. Since the absorbancy values are influenced by the amount of iodine and potassium iodide present in the mixture as well as the degree of dilution, tests were performed to determine which conditions were most suitable. In these tests, summarized in Fig. 1, varying degrees of "starch" conversion have been attained by using different concentrations of flour extract and the same hydrolyzing time. Because of the results obtained in these and other similar investigations, 0.0946 mg. iodine and 43.19 mg. of potassium iodide per 1 mg. "starch" employed in the hydrolyzing mixture were adopted for use in the colorimetric methods. The degree of dilution is also important and the dilution used was 40 ml. of water added to 10 ml. of diluted iodine solution and 2 ml. of hydrolysate, giving an absorbancy for BS ($= E_o$) of about 5.8 with the use of the Evans EEL Colorimeter.

A zero-order relationship was found by Hoskam (5) for the action of alpha-amylase up to 50% conversion and by Jongh (6) up to 20% conversion of erythrodextrin. With the amount of iodine solution used in the colorimetric method, a first-order reaction was found (Fig. 2) from 35 to 70% erythrodextrin conversion. Whether the reaction

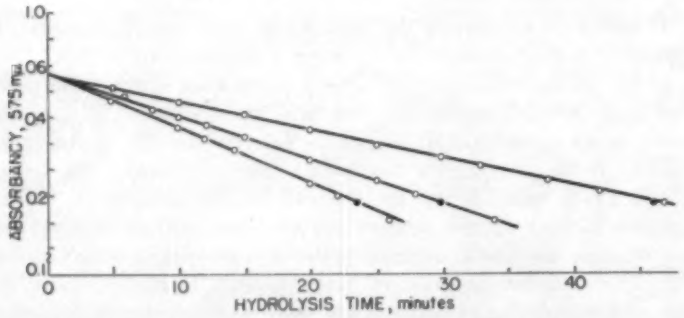

Fig. 2. Relation between absorbancy and time of hydrolysis by extracts of three different flours. Solid circles represent absorbancy at the end-point determined by Modified AACC Method IB.

proceeds according to the zero-order law with the concentration used and if so, how far, was not investigated in this work, since the absorbancy values corresponding to a starch conversion of less than 35% often were not reproducible. The absorbancy values corresponding to an erythrodextrin conversion of over 65 to 70% also were less reliable. Flour extracts which were not clear gave higher absorbancy values than expected, apparently because starch particles present in the flour extract influenced the readings.

Amylase Activity Values. The amylase activity values of wheat and rye flour or finely pulverized grains determined by the different meth-

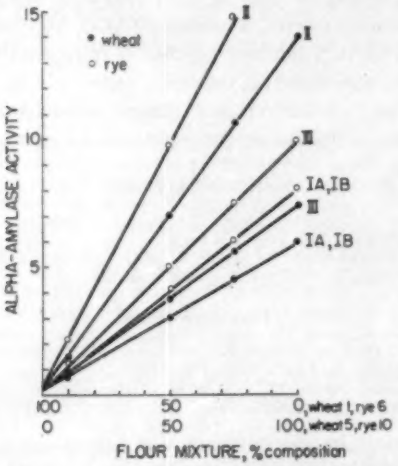

Fig. 3. Relation between alpha-amylase activity and composition of wheat and rye flour mixtures, respectively, determined by different methods. In Methods IA and IB, alpha-amylase activity is expressed in SKB units by multiplying the experimental values by the factors 1.0 and 0.7 respectively. In Method II, amylase activity is expressed in H units and in Method III, in V units.

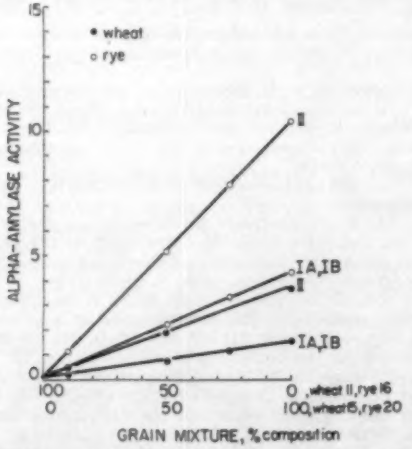

Fig. 4. Relation between alpha-amylase activity and composition of wheat and rye pulverized grain mixtures determined by different methods. In Methods IA and IB, alpha-amylase activity is expressed in SKB units by multiplying the experimental values by the factors 1.0 and 0.7 respectively. In Method II, amylase activity is expressed in H units.

ods are recorded in Figs. 3 and 4 respectively. From these and other tests the factors for transforming the values obtained into SKB units have been computed.

The reproducibility with Modified AACC Method I is about the same as with the AACC method (1). Good reproducibility also can be achieved with the colorimetric methods, especially in Method II. The values usually are evaluated from three absorbancy readings and corresponding times following the equation for a first-order reaction.

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CHARACTERIZATION OF INTERMEDIARY FRACTIONS OF HIGH-AMYLOSE CORN STARCHES[1]

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ABSTRACT

Starches from high-amylose corn of *du su*$_2$, *ae su*$_1$, *ae ae* types, and from commercial corn have been shown to possess a fraction intermediate between amylose and amylopectin, which has been isolated in yields of 8.7, 7.5, 6.6, and 4.5%, respectively. Separation is effected by fractionating out the amylose as either the 1-butanol or 1-nitropropane complex and then precipitating the intermediate fraction from the supernatant with 2-nitropropane. Intermediate fractions from these different starches are similar in iodine color, iodine blue value, iodine sorptive capacity, optical rotation, and rate of retrogradation. Those from the high-amylose starches have much lower viscosities and have possibly lower molecular weights than the fraction from commercial corn. All properties suggest that the intermediate fractions contain less highly branched molecules than does amylopectin and that the molecules are intermediate in shape between amylose and amylopectin.

It is well known from the work of Schoch (10,15) and others (6,11,20) that starches cannot be divided sharply into amylose and amylopectin but that these two major fractions shade into each other through an intermediate fraction. Fractions which are not intermediate but rather are more highly branched than amylopectin are also well known (2,4,5,14). Because of mounting interest in high-amylose corn starch, the characteristics of the intermediate fraction are here investigated with the expectation that increased information concerning this component will be of use in further understanding the properties of these starches and, perhaps, the manner of starch formation.

While various means are available for isolation of the intermediate fraction, the method chosen here is sequential precipitation of a starch dispersion, first with 1-butanol or 1-nitropropane and then with 2-nitropropane. This choice was based on the observation that 1-butanol or 1-nitropropane, on addition to a dispersion of normal corn starch, produces a precipitated fraction consisting of 26 to 28% of the starch with a high iodine sorption capacity, whereas 2-nitropropane produces a fraction of 28 to 31% with lower iodine sorption capacity (20). Differences in yield and iodine sorption capacities persist even when the isolated fractions are purified by recrystallization of the respective complexes. It is deduced that 2-nitropropane is a

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broad-spectrum complexing agent capable not only of complexing the linear amylose molecules but also some slightly branched molecules. Therefore, addition of 2-nitropropane to a starch dispersion from which amylose has been removed by means of 1-butanol or 1-nitropropane should lead to precipitation of the slightly branched molecular fraction.

Materials and Methods

Starches were isolated by conventional laboratory procedures from corn produced in the 1956 crop year. Starches of high amylose content were from corn of known genetic background (9) and were designated *du* su_2, *ae* su_1, and *ae ae*, containing 53, 64, and 66% amylose, respectively. The total starch content of the kernels approached that of normal corn. Before fractionation, the starch was extracted with methanol (8,16).

Each starch was fractionated by two procedures. In one, the amylose fraction was first separated with 1-butanol (17) and, after distillation of 1-butanol from the supernatant, the intermediate fraction was precipitated by addition of 2-nitropropane. The second fractionation procedure was conducted similarly, except that the amylose fraction was first separated with 1-nitropropane (20). High-amylose starches were not completely dispersed in hot water saturated with 1-butanol or 1-nitropropane. Therefore, these starches were pretreated by soaking in liquid ammonia (7) before being subjected to aqueous dispersion. The supernatant solution remaining after removal of the 1-butanol- or 1-nitropropane-amylose complex was concentrated slightly under reduced pressure to remove 1-butanol or 1-nitropropane. The solutions at pH 5.6–6.2 were adjusted to about 2% concentration (volume, 6 l.) and heated to 85°C.; 135 g. of 2-nitropropane were added. The vessel holding the dispersion was insulated by cloth wrappings, and the solution was allowed to cool slowly with stirring. After 48 hours, the mixture was centrifuged in a Spinco ultracentrifuge. The precipitate was redispersed to 2% concentration in water at 85°C., an excess of 2-nitropropane added, and the mixture allowed to cool slowly with stirring.

Iodine sorption capacity was measured potentiometrically by the procedure of Bates, French, and Rundle (3) as modified by Wilson, Schoch, and Hudson (22) and colorimetrically by the procedure of McCready and Hassid (12).

Osmotic molecular weights were determined on the acetylated fractions dissolved in chloroform using the Stabin-Immergut modification (18) of the Zimm-Myerson osmometer (23).

Intrinsic viscosities were measured in 1*N* potassium hydroxide solution in an Ostwald-Cannon-Fenske viscometer following the procedure of Lansky, Kooi, and Schoch (10).

The average number of D-glucose units per terminal nonreducing end group was determined by the method of Anderson, Greenwood, and Hirst (1) using cold potassium metaperiodate solutions.

Extent of retrogradation was measured in 0.5% aqueous solutions. One-half g. of sample was dissolved in 50 ml. of 1*N* potassium hydroxide solution at 25°C.; the solution was carefully neutralized with 1*N* hydrochloric acid solution and centrifuged. After 15 days, precipitated material removed by centrifugation was dissolved in 1*N* potassium hydroxide solution, and the concentration was determined by measurement of optical rotation. Optical rotations of intermediate fractions from high-amylose starches averaged $[\alpha]_D^{25} + 150°$.

Acetylation of fractions was accomplished in acetic anhydride-pyridine mixture (3.2:3.7 v/v) at 100°C. (21). Acetyl determinations done according to the method of Murray, Staud, and Gray (13) showed that the fractions were fully acetylated.

Results and Discussion

The amounts of intermediate fractions obtained from the various high-amylose starches and from starch of normal commercial hybrid corn are in the range of 8.7 to 4.5%. Thus, the yields from starches of *du* su_2, *ae* su_1, and *ae ae* lines (Purdue high-amylose genotypes) and normal corn varieties are 8.7, 7.5, 6.6, and 4.5%, respectively. The fractions are obtained in quite pure condition, as evidenced by the very slight loss observed on recrystallization and the finding that the acetates, when dissolved to 1% concentration in chloroform, precipitate within a narrow range of hexane concentration when this nonsolvent is slowly added with stirring. Intermediate fractions precipitated by 2-nitropropane following removal of amylose with either 1-butanol or 1-nitropropane are almost identical in all properties. Therefore, only the fractions obtained with the use of 1-butanol and 2-nitropropane are described here.

As might possibly be expected, the properties of the fractions from the different starch types are similar and are intermediate between those of amylose and amylopectin (Table I). All intermediate fractions give a deep-blue color with iodine, visually similar to the amylose-iodine color. However, depth of color is less, as shown by the lower blue values (12) and the lower amount of iodine taken up on potentiometric titration. In addition, the maximum absorbance for the iodine solution of the intermediate fraction is at 588–600 mμ,

TABLE I

PROPERTIES OF STARCH FRACTIONS

Starch Fraction	Coloration by Iodine	Blue Values (Absorbance)	Iodine Absorbed per g. Starch Fraction	Average Number of D-Glucose Units per Nonreducing End Group	Limiting Viscosity in Normal Potassium Hydroxide Solution	Retrogradation within 15 Days	Yield of Fraction	$[\alpha]_D^{25}$
			mg.			%	%	
Intermediate from normal starch	blue	0.26	58	41	1.26	26	4.5	+153
Intermediate from du su_2 starch	blue	0.25	51	30	0.375	19	8.7	+149
Intermediate from se su_1 starch	blue	0.26	57	40	0.308	28	7.5	+151
Intermediate from se se starch	blue	0.31	61	47	0.195	34	6.6	+150
Amylose from normal starch	blue	0.45	196	409	1.04	98	..	+159
Amylopectin from normal starch	reddish-purple	0.07	3	25	1.48	3	..	+155

which is between the values for amylopectin (540 mμ) and amylose (650 mμ) (19).

Periodate end-group determinations show that all intermediate fractions have roughly the same degree of branching with approximately one nonreducing end group for each 30—47 D-glucose units. The intermediate fraction is much less branched, therefore, than amylopectin, having, on the average, only 64% of the branching frequencies of amylopectin.

Osmotic molecular-weight determinations on the triacetates of the amylose and intermediate fractions from commercial corn starch gave values of 303,000 and 93,000, respectively.

According to viscosity measurements, the intermediate fractions of high-amylose starches have molecular weights which are lower than those of the intermediate fraction of commercial corn starch. The bearing of this on possible schemes for starch biosynthesis is intriguing.

Intermediate fractions, even though possessing frequent branches and, in most instances, low molecular weights, are capable of retrogradation, though at a slow rate.

In view of the similarity of optical rotations of amylose and amylopectin in alkaline solution, the equivalent rotary power of these intermediate fractions is expected.

The iodine sorption and periodate values indicated the intermediate fractions to be close to amylopectin in structure but with longer branch length and perhaps smaller molecular size.

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PROTEINS OF WHEAT AND FLOUR. EXTRACTION, FRACTIONATION, AND CHROMATOGRAPHY OF THE BUFFER-SOLUBLE PROTEINS OF FLOUR[1]

J. H. COATES[2] AND D. H. SIMMONDS

ABSTRACT

Six major protein fractions were identified following chromatography on DEAE-cellulose of the proteins extracted by 0.01*M* sodium pyrophosphate (at pH 7) from each of two flour samples differing widely in protein content and baking performance. The two flours appeared to differ in the relative amounts of certain of the components present. Two of the fractions were resolved further by rechromatography and by electrophoresis, giving a total of ten components so far recognized in these extracts.

One fraction (peak A), containing both protein and carbohydrate, passed unretarded through the DEAE-cellulose column. On hydrolysis, the carbohydrate component of peak A yielded arabinose and xylose, together with lesser amounts of galactose. Electrophoresis and further chromatography of peak A on carboxymethyl-cellulose (CM-cellulose) demonstrated it to be heterogeneous and to consist of at least three components. Peaks D, E, and F were eluted from the DEAE-cellulose column in the presence of increasing concentrations of sodium chloride. Electrophoresis experiments indicated the presence of at least five components in this group of proteins with the following approximate mobility values: A, $0\text{–}3 \times 10^{-6}$; D, $19\text{–}22 \times 10^{-6}$; E, $26\text{–}27 \times 10^{-6}$; F, $43\text{–}50 \times 10^{-6}$ $cm^2\ sec^{-1}\ volt^{-1}$. Peaks D, E, and F were rechromatographed on DEAE-cellulose to yield single symmetrical peaks. Components J and K were eluted by 0.05*N* acetic acid and 0.1*N* sodium hydroxide, respectively. Peaks D and E, F and K have a lower amide and glutamic acid content than peak A and are higher in arginine, aspartic acid, glycine, leucine, and tyrosine.

As part of a comprehensive study of the proteins of developing and mature wheat grain, methods were required for the extraction, separation, and identification of individual proteins. Chromatography on columns of cellulose ion-exchangers was therefore investigated for this purpose. This paper describes the extraction and chromatographic procedures used and the results of electrophoretic studies on the products so obtained.

Materials and Methods

Two wheat samples, varieties Tichborne (sample 1) and Broughton (sample 5), were milled on a Buhler laboratory mill to 72 and 70% extraction, respectively. The resulting flour samples contained 2.61 and 1.60% nitrogen on a dry-weight basis. The two flours dif-

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fered considerably in baking behavior and in response to bromate addition when subjected to the standard baking procedure developed by the Bread Research Institute, Sydney. In this procedure two doughs are prepared from each flour consisting of flour (120 g.) to which are added 68 ml. of a liquor containing yeast (2.4 g.), sodium chloride (2.4 g.), and ammonium chloride (0.06 g.). To the unbromated dough 6 ml. water are added; the bromated dough is mixed with 0.02% potassium bromate solution (6 ml.; equivalent to 10 p.p.m. on the weight of flour taken). After mixing, proofing, etc., under controlled conditions, the loaves are baked at 510°F. (266°C.) and scored according to volume (possible mark, 36%), external appearance (20%), crumb texture (30%), and crumb color (14%). In this test flour 1 gave a medium-extensible dough, yielding an unbromated loaf of volume 550 ml. and a bromated loaf of volume 600 ml. Its quality rating was 63% without and 79% with the addition of bromate, and it therefore showed a normal response to this ingredient. Flour 5 yielded a strong, stable dough giving both unbromated and bromated loaves of volume 500 ml. Its quality score was 64% without and 65% with the addition of bromate, and it therefore showed a zero or negative response to this ingredient.

Preliminary Studies. Preliminary studies showed that the presence of lipids in dilute sodium pyrophosphate extracts (24) of disrupted wheat grain caused considerable interference during subsequent fractionation attempts with ammonium sulfate and ethanol precipitation. The lipids were successfully removed by preliminary treatment with water-saturated 1-butanol (17,23,24). Examination of the fractions so obtained by moving-boundary electrophoresis showed them to be markedly heterogeneous. Attempts were therefore made to fractionate them further on columns of cellulose ion-exchangers (33,37).

Extraction of Buffer-Soluble Flour Proteins. Both flour samples were extracted side by side according to the procedure summarized in Diagram I. All operations were carried out in a cold room at 4°C.

After preliminary treatment in a Waring Blendor with water-saturated 1-butanol, the flour samples were extracted several times with sodium pyrophosphate solution (0.01*M*, pH 7.0), followed by several extractions with acetic acid (0.05*N*, pH $\simeq$ 3.5). The nitrogen extracted from each flour at each stage is shown in Diagram I, expressed as a percentage of the total nitrogen in that flour.

The yellow-colored butanol extracts contained considerable amounts of lipid but only 0.4 to 0.5% of the total nitrogen. Since other studies (23,24) have shown that the nitrogenous constituents

Diagram I. Extraction Procedure for Flour Samples 1 and 5

100 g. FLOUR 1 AND 5

Homogenized 5 minutes with 200 ml. water-saturated butanol; centrifuged.

RESIDUE stirred 1.5 hours with 200 ml. 0.01*M*, pH 7.0 sodium pyrophosphate; centrifuged.

BUTANOL EXTRACT
(1): 0.50% (5): 0.36%

RESIDUE stirred 1 hour with 200 ml. 0.01*M*, pH 7.0 sodium pyrophosphate; centrifuged.

First 0.01*M* PYROPHOSPHATE EXTRACT
(1): 6.92% (5): 10.56%

RESIDUE: two further extractions with 0.01*M* sodium pyrophosphate; centrifuged between each.

Second 0.01*M* PYROPHOSPHATE EXTRACT
(1): 5.20% (5): 6.96%

Third 0.01*M* PYROPHOSPHATE EXTRACT
(1): 2.64% (5): 4.03%

Fourth 0.01*M* PYROPHOSPHATE EXTRACT
(1): 2.06% (5): 3.89%

RESIDUE stirred 1.5 hours with 200 ml. 0.05*N* acetic acid; centrifuged.

Total N extracted into 0.01*M* sodium pyrophosphate
(1): 16.82% (5): 24.64%

RESIDUE stirred 1 hour with 200 ml. 0.05*N* acetic acid; centrifuged

First 0.05*N* ACETIC ACID EXTRACT
(1): 32.76% (5): 27.35%

RESIDUE: two further extractions with 0.05*N* acetic acid; centrifuged between each.

Second 0.05*N* ACETIC ACID EXTRACT
(1): 28.74% (5): 21.19%

Third 0.05*N* ACETIC ACID EXTRACT
(1): 4.99% (5): 6.86%

Fourth 0.05*N* ACETIC ACID EXTRACT
(1): 1.50% (5): 2.92%

RESIDUE:
(1): 14.65% (5): 16.69%

Total N extracted into 0.05*N* acetic acid:
(1): 67.99% (5): 58.32%

Total N recovered:
(1): 100.7% (5): 99.3%

were mainly phospholipids, the butanol extracts were not further examined.

The combined sodium pyrophosphate extracts of each flour contained 16.8% (flour 1) and 24.6% (flour 5) of the total nitrogen. They were separately dialyzed for 2 days against three changes of glycine-sodium hydroxide buffer (0.006*M*, pH 9.5, 200 ml.) on a rocking dialyzer at 4°C.

Chromatography of Proteins. Diethylaminoethyl-cellulose (DEAE-cellulose)[3] was washed with 0.1*N* sodium hydroxide and then with distilled water. It was then poured into a glass or Perspex column to form a bed 2 cm. in diameter and 15 cm. high. The column was washed successively with 500 ml. glycine-sodium hydroxide (0.2*M*, pH 9.5) and 1,500 ml. glycine-sodium hydroxide buffer (0.006*M*, pH 9.5). The dialyzed pyrophosphate extract containing 25–50 mg. of protein nitrogen was applied to the top of the prepared column, and the effluent was collected in 4-ml. aliquots by means of a fraction collector. All operations were carried out in a cold room at 4°C.

Carboxymethyl-cellulose (CM-cellulose) was prepared by treatment of Whatman standard cellulose powder[4] with chloroacetic acid, followed by thorough washing with 10% acetic acid and distilled water (33). The ion-exchanger was then stirred with 0.1*N* sodium hydroxide (20 volumes) and 0.1*N* hydrochloric acid (20 volumes), followed by thorough washing with distilled water. It was suspended in acetic acid-sodium hydroxide buffer (20 volumes; 0.5*M*, pH 4.1) and poured into a column 2 cm. in diameter and 15 cm. high. Acetate buffer (0.5*M*, pH 4.1; 500 ml.) was passed through the column, followed by 1,500 ml. of acetate buffer (0.005*M*, pH 4.1). The protein solution (30 ml. containing 250 mg., peak A), dialyzed for 2 days against three changes of acetate buffer (0.005*M*, pH 4.1; 2,000 ml.), was then applied to the top of the prepared column. The column was washed successively with 200 ml. acetate buffer (0.005*M*, pH 4.1) and 200 ml. acetate buffer (0.005*M*, pH 4.1) containing urea (final concentration 4*M*), followed by a gradient to acetate buffer (0.005*M*, pH 4.1) containing urea (final concentration 4*M*) and sodium chloride (1*M*). The effluent was collected in 4-ml. aliquots on a fraction collector.

Analytical Methods. Absorbance values of the effluent samples were measured at 280 $m\mu$ with a Uvispek spectrophotometer. Nitrogen was estimated by a micro-Kjeldahl procedure. Hydrolysis of the samples with twice-redistilled, constant-boiling hydrochloric acid was

[3] Eastman Kodak Co., Rochester, N. Y.
[4] W. and R. Balston, Ltd., London, England.

carried out in evacuated sealed tubes for 20 hours at 110°C.

Amino acids were estimated by the ion-exchange chromatographic procedure of Moore, Spackman, and Stein (22); the automatic apparatus of Simmonds and Rowlands (34,35) was used.

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (13), and also as micro-Kjeldahl-determined nitrogen multiplied by the factor 5.7.

Pentoses were estimated according to Dische (7) and hexoses by the method of McCready, Guggolz, Silvera, and Owens (16).

Electrophoresis Studies. Moving-boundary electrophoresis was carried out at 1.8° ± 0.05°C. using a Perkin-Elmer apparatus (model 38 A) with an open-cell assembly of volume approximately 2 ml. A standard glycine-sodium hydroxide buffer (ionic strength 0.1, pH 9.5) was employed as solvent. Protein solutions (approximately 4 ml.) were dialyzed against two changes of about 500 ml. of buffer at 4°C. for 48 hours before electrophoresis. The pH of both protein solution and external buffer was measured; the former was recorded as the pH at which electophoresis was carried out.

Refractive index gradients in both limbs of the electrophoresis cell were observed with a schlieren optical system in which the knife edge was replaced by a phase plate having a human hair attached along the phase border. The positions of the maximal ordinates of the schlieren patterns were measured relative to the shadow of a wire fixed across the cell mask. The cell current was measured with an avometer. All conductivity measurements were carried out at the temperature of electrophoresis. Mobilities are given for the descending limb only. Under the experimental conditions used, all proteins migrated toward the anode.

Ultracentrifuge Studies. A Spinco model E analytical ultracentrifuge was used.

Results

Chromatography of Flour Proteins. Figure 1 shows a typical trace of absorbance vs. effluent volume obtained when a pyrophosphate extract of flour 1 containing 55 mg. of nitrogen was loaded onto a 2-cm. by 15-cm. column of DEAE-cellulose.

Material designated peak A was not retained by the column and was washed out with a further 400 ml. of glycine-sodium hydroxide buffer (0.006*M*, pH 9.5). A linear gradient to 0.3*M* sodium chloride in glycine sodium hydroxide (0.006*M*, pH 9.5) was then started.

The retention of peaks D and E appears to be very sensitive to the concentration of sodium chloride in the eluting buffer; optimum

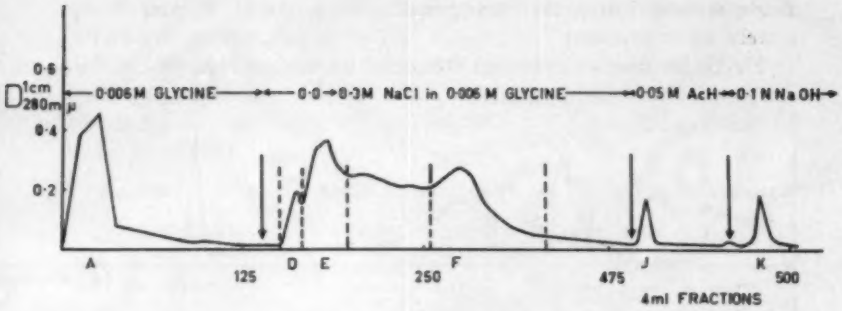

Fig. 1. Chromatography of 0.01*M* sodium pyrophosphate extract of flour 1 on DEAE-cellulose (5 g.; 2-cm. by 15-cm. column). Loading approximately 55 mg. of protein nitrogen in 50 ml. dialyzed solution. Column equilibrated with glycine-sodium hydroxide buffer (0.006*M*, pH 9.5).

resolution was obtained with a slow, continuous gradient in which 800 ml. of the starting eluent (glycine-sodium hydroxide; 0.006*M*, pH 9.5) and 800 ml. of the limit eluent (0.3*M* sodium chloride in glycine-sodium hydroxide, 0.006*M*, pH 9.5) were placed in each arm of the gradient device. Any discontinuity in the gradient was reflected by the appearance of artifact peaks in the effluent. This occurred if the gradient was changed in steps from 0.0 → 0.2*M* sodium chloride to 0.2 → 0.3*M* sodium chloride. No further components could be eluted by increasing the sodium chloride gradient to 1.0*M*. The effect of pH was not studied exhaustively, but poor separations were obtained at pH 8.5.

Regeneration of the column with 0.1*N* sodium hydroxide led to the desorption of two further components, J and K, one of which could be eluted with 0.05*N* acetic acid, or by running a further gradient from 0.006*M* glycine to 0.1*N* HCl prior to regeneration with sodium hydroxide (see Fig. 1). Flours 1 and 5 gave similar elution patterns, varying mainly in the relative proportions of peaks D, E, and F. As a guide, peaks A, D + E, and F represented about 30–40, 25–35, and 15–20% dry weight, respectively, of the pyrophosphate-extractable material. The remaining components were present in small amounts only.

Material for rechromatography, electrophoresis, ultracentrifuge studies, and amino acid analysis was obtained by combining fractions of the column effluent from various runs in a manner similar to that indicated in Fig. 1. Figure 2 illustrates the purification achieved by rechromatography (on DEAE-cellulose) of appropriately combined effluent fractions. After two further chromatographic separations,

single symmetrical peaks corresponding to peaks D, E, and F separately were obtained.

Electrophoresis of Fractions Obtained by Chromatography. Before

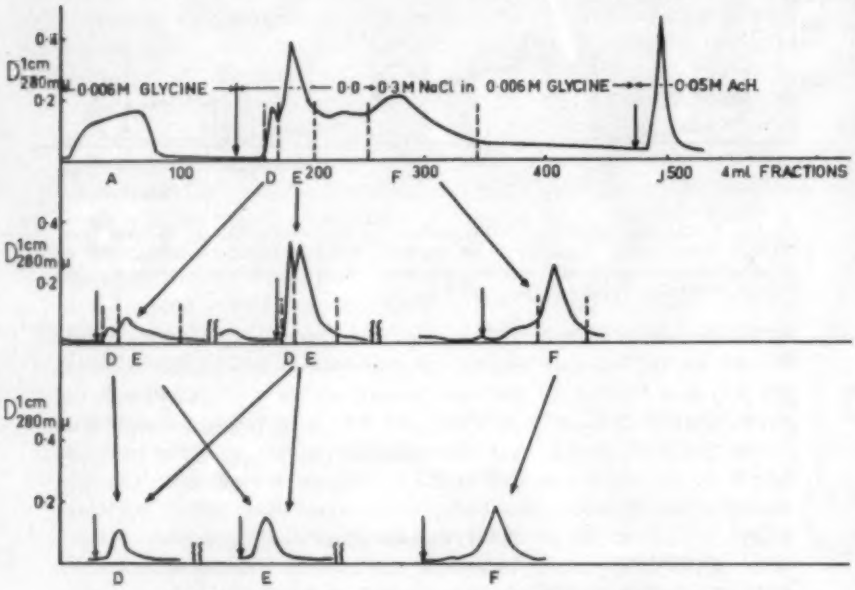

Fig. 2. Rechromatography of appropriately combined material from peaks D, E, and F on DEAE-cellulose. Column loading and buffers as in Fig. 1.

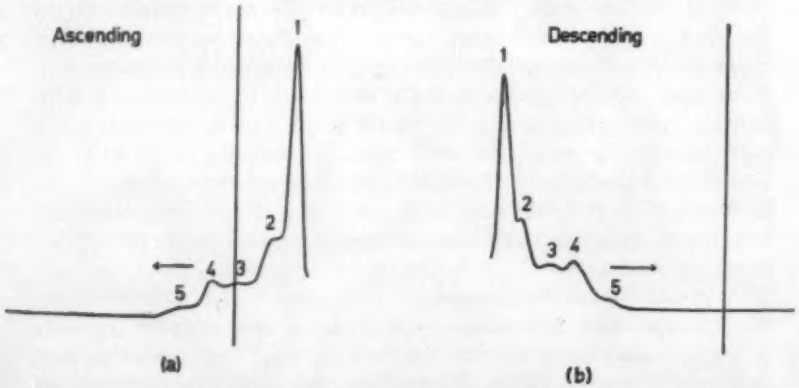

Fig. 3. Electrophoresis of sodium pyrophosphate extract of flour 1 in glycine-sodium hydroxide buffer, pH 9.98 (a) ascending limb, (b) descending limb after 2 hours. Field strength 3.62 volts per cm.

chromatography the pyrophosphate extract of flour 1 gave the electrophoresis pattern shown in Fig. 3.

The large peak close to the initial boundary moved so little that it was not possible to ascribe to it a meaningful mobility value. Four further peaks were observed having the mobilities summarized in Table I.

TABLE I

MOBILITY VALUES OF THE MAJOR PROTEIN COMPONENTS SHOWN IN THE ELECTROPHORESIS EXPERIMENT OF FIG. 3[a]

PROTEIN COMPONENT	ASCENDING LIMB	DESCENDING LIMB
1	0.19	1.08
2	12.59	8.03
3	26.53	20.44
4	36.58	35.95
5	53.64	42.79

[a] Values are expressed as $cm.^2\ volt^{-1}\ sec.^{-1} \times 10^{-5}$.

Electrophoresis experiments were also carried out on the main fractions obtained after chromatography on DEAE-cellulose. The material which was not retained by the column (peak A) gave rise to two electrophoretic components with mobilities of 3 and 12 $cm.^2$

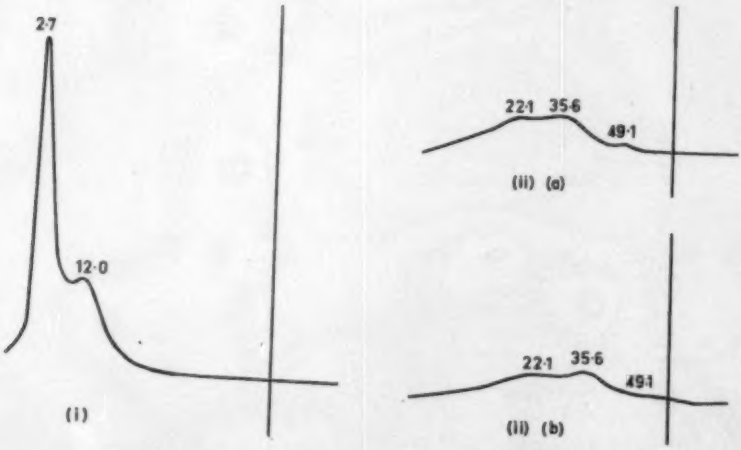

Fig. 4. Electrophoresis of sodium pyrophosphate extract of flour 1. (i) Material passing unretarded through 2-cm. by 15-cm. DEAE-cellulose column (peak A). Electrophoresis carried out in glycine-sodium hydroxide buffer. pH 9.40, descending limb after 5 hours. Field strength 3.67 volts per cm. (ii) Material eluted by 0.3*M* sodium chloride from 2 cm. by 15 cm. DEAE-cellulose column. Electrophoresis carried out in glycine-sodium hydroxide buffer. pH 9.28, descending limb (a) after 3 hours, (b) after 4 hours. Field strength 3.35 volts per cm.

volt^{-1} sec.$^{-1}$ $\times$ 10^{-6}, respectively, as shown in Fig. 4, i.

Electrophoresis of the material adsorbed by the DEAE-cellulose column and eluted by 0.3*M* sodium chloride (consisting of a mixture of peaks D, E, and F, Fig. 1) showed the presence of three components having mobilities of 22, 36, and 49 cm.^{2} volt^{-1} sec.$^{-1}$ $\times$ 10^{-6} (Fig. 4, ii).

When effluent fractions combined from the individual peaks marked D, E, and F in Fig. 1 were separately concentrated and examined by electrophoresis, a total of six components were detected as shown in Fig. 5.

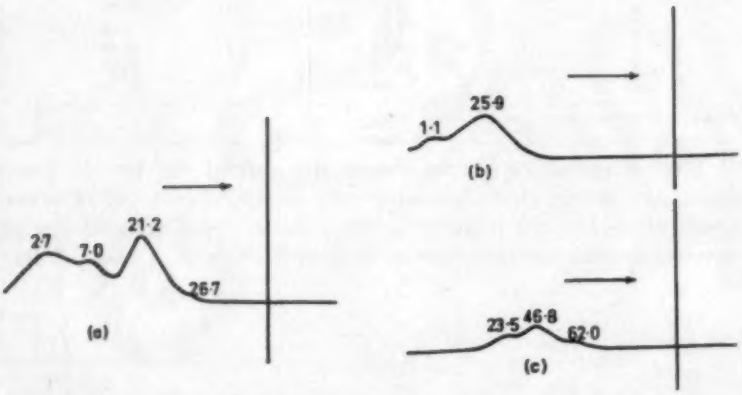

Fig. 5. Electrophoresis of sodium pyrophosphate extract of flour 1. Material eluted by 0.0–0.3*M* sodium chloride gradient from 2-cm. by 15-cm. DEAE-cellulose column as illustrated in Fig. 1. (a) Concentrate from area marked peak D (Fig. 1). Glycine-sodium hydroxide buffer, pH 9.46, descending limb after 5¼ hours. Field strength 3.64 volts per cm. (b) Concentrate from area marked peak E (Fig. 1). Glycine-sodium hydroxide buffer, pH 9.51, descending limb after 2¼ hours. Field strength 3.77 volts per cm. (c) Concentrate from area marked peak F (Fig. 1). Glycine-sodium hydroxide buffer, pH 9.42, descending limb after 2½ hours. Field strength 3.80 volts per cm.

The mobility values calculated from these electrophoretic runs are given in Table II.

Preliminary analysis of peak E and peak F material in the ultracentrifuge has shown in both the presence of only two components, having sedimentation coefficients of approximately 5S and 2S, respectively. In view of the greater resolution attainable by electrophoresis, further ultracentrifuge work has been deferred until electrophoretically homogeneous components have been prepared.

Material eluted from the DEAE-cellulose column by 0.05*N* acetic acid, or by 0.1*N* hydrochloric acid at pH 1.5–2 (peak J), contained

TABLE II

MOBILITY VALUES OF COMPONENTS IN FRACTIONS D, E, AND F ELUTED FROM DEAE-CELLULOSE[a]

SAMPLE	MOBILITY VALUES[b] AND APPROXIMATE PROPORTIONS[c]						
	0.3	7-13	19-22	26-27	36	43-50	60
Total extract	+++	++	++	−	++	+	−
Eluted by 0.3*M* NaCl	+	−	++	−	++	+	−
Peak A	+++	++	−	−	−	−	−
Peak D	+	+++	++	+++	−	−	−
Peak E	+	−	−	+++	−	−	−
Peak F	−	−	−	++	−	+++	+
Peak J	++	−	−	−	−	++	−

[a] Calculated from electrophoretic analyses illustrated in Fig. 5.
[b] $cm.^2\ sec.^{-1}\ volt^{-1} \times 10^{-5}$.
[c] +, small amount only; ++, moderate amount present; +++, main component; −, not detected.

two electrophoretic components with mobilities of 4 and 43 $cm.^2\ volt^{-1}\ sec.^{-1} \times 10^{-6}$ respectively.

Further Purification and Chemical Properties of Peak A. Rechromatography of peak A on DEAE-cellulose equilibrated at pH 9.5 with 0.005*M* borate buffer yielded two major components, A_1 and A_2; the former passed unretarded through the column, and the latter was eluted in the position of peaks D, E, and F (Fig. 1) by 0.005*M*,

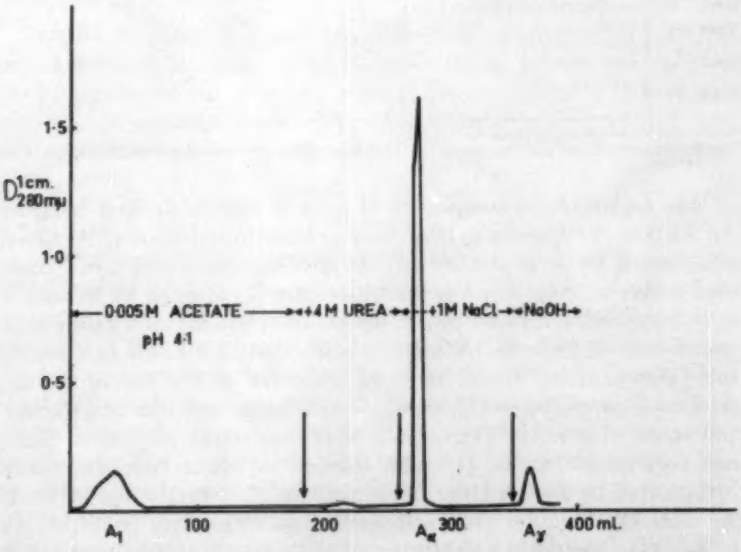

Fig. 6. Rechromatography of peak A on CM-cellulose equilibrated with acetate buffer (0.005*M*, pH 4.1). Loading: 250 mg. of lyophilized peak A in 30 ml. dialyzed solution.

pH 9.5 borate buffer containing 0.3*M* sodium chloride. Peak A_1 gave positive tests for protein (13) and carbohydrate (Molisch); Peak A_2 contained protein and gave only a very weak Molisch test. The borate treatment has therefore separated from peak A, a component corresponding in chromatographic mobility to peaks D, E, and F in Fig. 1.

Further evidence for the heterogeneity of peak A has come from rechromatography on columns of CM-cellulose. At pH 4.1 in 0.005*M* sodium acetate containing 4*M* urea, three main components were observed, as shown in Fig. 6.

Peak A_1 passed straight through the column; peak A_α was eluted by 1*M* sodium chloride dissolved in 0.005*M* sodium acetate containing 4*M* urea; peak A_γ was eluted by 0.1*N* sodium hydroxide. All peaks gave positive protein and Molisch tests and contained the amounts of protein (N × 5.7) and carbohydrate (expressed as xylose, Dische, 7) shown in Table III.

TABLE III

COMPOSITION OF PEAK A AND ITS SUBFRACTIONS PREPARED FROM FIGURE 1[a]

COMPONENT	PROTEIN (N × 5.7)	CARBOHYDRATE (DISCHE, 7)	TOTAL ACCOUNTED FOR
Original peak A (from DEAE-cellulose column)	40.6	49.6	90.2
Peak A_1 from CM-cellulose column	6.4	87.2	93.6
Peak A_α from CM-cellulose column	69.0	15.4	84.4
Peak A_γ from CM-cellulose column	++++	+	—

[a] Results are expressed as percentage dry weight.
[b] Insufficient material available for analysis. + indicates relative amounts of protein and carbohydrate present.

The carbohydrate component of peak A and its derived fractions A_1, A_α, and A_γ appears to be mainly pentose in nature as determined colorimetrically with orcinol (7). It gave comparatively little color with anthrone (16). The absence of starch was indicated by its failure to give any color with iodine. Paper chromatography of a dilute acid hydrolysate of peak A (1*N* sulfuric acid, 110°C., 6 hours in a sealed tube) demonstrated the presence of arabinose and xylose in the approximate proportions 1:2, together with lesser amounts of galactose and traces of glucose. The carbohydrate component of peak A therefore corresponds to the pentosan fraction of wheat flour previously investigated by Pence, Elder, and Mecham (27), Perlin and co-workers (8,31,32), Simpson (36), and Smith and co-workers (10,11,18–21). It is likely that the traces of glucose observed are due to slight contamination with starch. The presence of galactose confirms a similar observation made by Simpson (36) and Ford and Peat (9).

Fractions separately combined from the areas marked A, D + E, F, and K in the chromatographic experiment of Fig. 1 have also been subjected to amino acid analysis. The results, determined on protein fractions from flour 1, are summarized in Table IV.

TABLE IV

AMINO ACID COMPOSITION OF PEAKS A, D + E, F, AND K DERIVED FROM FLOUR SAMPLE 1[a]

AMINO ACID	PROTEIN FRACTIONS			
	A	D+E	F	K
Alanine	3.3	6.2	6.1	4.9
Amide	14.8	10.7	8.3	12.4
Arginine	11.0	18.6	19.6	17.1
Aspartic acid	4.1	6.4	7.1	6.3
Glutamic acid	16.1	12.5	10.7	8.7
Glycine	3.6	6.8	7.0	6.2
Histidine	3.9	6.2	6.4	6.1
Isoleucine	2.4	2.9	3.2	3.1
Leucine	4.0	6.3	7.1	5.3
Lysine	4.4	6.4	6.7	6.7
Phenylalanine	2.9	1.8	2.1	2.7
Serine	3.4	4.5	3.8	3.4
Threonine	2.7	3.0	3.4	2.7
Tyrosine	1.2	2.6	3.6	2.1
Valine	2.6	5.4	5.2	4.3

[a] Amino acid nitrogen is expressed as a percentage of the total nitrogen present in the sample after hydrolysis.

It will be noted that peak A, containing approximately 45% of protein, differs markedly from the other three protein fractions in having an amide and glutamic acid content approaching that of gluten (28). Its content of alanine, arginine, aspartic acid, glycine, histidine, leucine, lysine, tyrosine, and valine is less than that of the other peaks.

Discussion

Moving-boundary electrophoresis was used in this study as a qualitative tool to evaluate the effectiveness of substituted cellulose ion-exchange columns for the separation of soluble wheat proteins. Figure 3 shows that the patterns obtained in both ascending and descending limbs are enantiographic within the limits imposed by the electrophoretic technique (1). This supports the assumption that these proteins migrate in an electric field in a manner uncomplicated by interactions and permits identification of the macromolecular components of the column effluents with specific components in the electrophoretic patterns. Such components may or may not correspond to discrete protein species in terms of other physicochemical criteria. Additional components may well be detected by further electrophoresis studies over a wider range of pH and ionic strength than

those reported here. The mobility values quoted in Table II show reasonable agreement between ascending and descending limbs. Because of the complexity of the pattern, first-moment calculations for each peak could not be carried out, and apparent maximum ordinates were therefore used. This led to exaggerated differences in mobility values between ascending and descending limbs. This lack of precision in the mobility measurements was shown in the plots of distance moved against time and also in the lack of complete reproducibility of mobility values from run to run. For this reason, the ranges of values observed have been quoted in Table II. However, the over-all shapes of the electrophoretic patterns were fully reproducible.

During concentration of the column effluent fractions by freeze-drying, a small quantity of insoluble material, possibly denatured protein, was formed. However, its removal by centrifugation of the final solutions used for electrophoresis is unlikely to have substantially affected the proportions of components observed. Further factors that complicated the interpretation of the results are the small but noticeable variations in pH, which affect the electrophoretic mobility values calculated on similar protein fractions from different chromatographic separations, and the tendency for any discontinuities in the salt gradient to be reflected as small peaks in the elution pattern.

Column chromatography on DEAE-cellulose has clearly split the pyrophosphate-soluble flour proteins into three main groups: 1) Material not retarded by the column – peak A; 2) material adsorbed by the column but eluted by low concentrations of sodium chloride – peaks D, E, and F; and 3) material eluted from the column only under extremes of pH – peaks J and K.

Electrophoresis of peak A consistently showed the presence of two components: a major component which remains practically stationary, and a minor peak with a mobility of about 12 $cm.^2\ volt^{-1}\ sec.^{-1} \times 10^{-6}$. It is possible that the stationary peak corresponds to the carbohydrate-rich material which passes unretarded through a CM-cellulose column (peak A_1, Fig. 6) and the minor component to the protein-rich peak A_α (Fig. 6). It seems significant that rechromatography of peak A on borate-DEAE-cellulose splits from the original mixture a component which runs in a position similar to that of peaks D, E, and F. It is possible that the pentosan interacts with members of this group to yield products which are stable to chromatography. The stability and strength of this interaction are matters requiring further investigation.

With respect to the second group of proteins, those adsorbed by the DEAE-cellulose column and eluted in the presence of sodium chloride, examination of Table I and Fig. 1 shows that, in general, components with low electrophoretic mobilities are eluted by low salt concentrations, whereas components with large electrophoretic mobilities are eluted at higher salt concentrations. The ultracentrifuge evidence indicates that the protein molecules comprising peaks D, E, and F do not differ greatly in their sedimentation properties. If their molecular weights are also similar, they will, in addition, have frictional coefficients of the same order. Under these conditions, the mobility of a given electrophoretic component will be approximately proportional to its effective charge under the conditions of pH ($\simeq$ 9.5) and ionic strength (0.1) used. Since the chromatographic analysis is conducted at a constant pH up to the elution of peak F, neither the adsorbed proteins nor the DEAE-cellulose will gain or lose hydrogen ions. However, the effective charges of both the column material and the adsorbed proteins will be reduced by the collapse of their ionic double layers as the ionic strength increases. If the adsorption of the proteins is purely electrostatic, the slow decrease in effective charge should result in the species of lowest charge desorbing from the column at lowest salt concentrations; whereas those of greater charge will require a higher ionic strength to reduce their effective charge to the point of desorption. If a particular protein has a very high charge at the pH of the experiment, increase in ionic strength may never collapse the ionic double layer sufficiently to permit desorption. Such appears to be the case with peaks J and K which can only be eluted by raising the pH until the removal of hydrogen ions from the DEAE-cellulose removes its charge completely.

It is interesting to compare the procedures and results described in this paper with the many methods which have been proposed for the extraction and separation of the proteins contained in the wheat grain and its milled products (2–4,6,12,14,15,25). Many albumin components were observed by Pence and co-workers (26,29,30) with the use of paper electrophoresis, and Danielson (5) separated two different globulins in the ultracentrifuge. In our hands, paper electrophoresis has yielded streaky patterns inferior to, but supporting in general results, the patterns obtained by solution electrophoresis. The few ultracentrifugal analyses carried out supported the electrophoresis results, but again the resolution into individual components was inferior. However, all results indicate that a complex mixture of proteins is extracted from flour by salt solutions at neutral pH values. Replicate chromatographic analyses on DEAE-cellu-

lose have given consistently reproducible patterns showing the presence of some six components. Electrophoresis and rechromatography have increased this total to at least ten, and further studies may well reveal the presence of yet additional minor components. Much work will be required to determine the exact relationship of these components to those reported elsewhere in the literature.

The authors' experience has been that wheat proteins are particularly sensitive to denaturing influences such as high temperatures and pH values. The extraction procedure developed (Diagram I) attempts to overcome these difficulties. Extraction with sodium pyrophosphate followed by acetic acid allowed a clear distinction to be made between the two main groups of proteins present. Prompt fractionation of the pyrophosphate extract on DEAE-cellulose gave protein fractions more clearly resolved than those attainable by salt fractionation. Furthermore, the techniques can be scaled up or down to suit the quantities of material available for examination. Thus, microscale studies are being initiated on developing wheat endosperm and to investigate differences in the protein composition of different flours. Large-scale preparation of individual proteins is also being undertaken, so that their physical and chemical properties can be examined and a study of their role in the baking process can be made.

Acknowledgments

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THE OXIDATION OF WHEAT FLOUR

I. Measurement of Sulfhydryl Groups [1]

BETTY SULLIVAN, LELAND DAHLE, AND ELOF LARSON

ABSTRACT

Although *p*-chloromercuribenzoate (PCMB) reacts specifically with free sulfhydryl groups, these groups can still be measured in the presence of PCMB by amperometric titration with mercuric chloride. Mercuric chloride apparently displaces the PCMB from –SH groups, the mercuric chloride possessing a stronger affinity for the –SH groups than PCMB. This provides a useful technique for protecting –SH groups during measurement and results in higher values.

The –SH content of flour is about equally divided between the water-soluble and the gluten proteins. No –SH has been found in the lipid or starch fraction of flour. The lipids of germ do contain sulfhydryl groups.

The maturing of flour is one of the most challenging and intricate problems in the field of cereal chemistry. The present status of the subject has been reviewed recently (10,13), It is generally believed that sulfhydryl groups are involved in the improvement of the physical properties of flour by aging and by maturing agents, but, in spite of the large amount of work on the subject, the entire mechanism remains obscure.

It is well known that –SH-containing compounds, such as glutathione, cysteine, and thioglycollic acid, soften the gluten and decrease the viscosity, plasticity, and mixing time of flour doughs (10,13). Free gluthathione is found in significant amounts in wheat germ (0.2 to 0.5% as cysteine) and to a lesser degree in bran and low-grade flour, but none is found in shorter-extraction flours. The nature of the –SH-containing substances in flour is not known. Presumably they are

[1] Manuscript received September 27, 1960. Contribution from the Research Laboratories of Russell Miller-King Midas Milling Company, Minneapolis, Minn.

cysteine residues of the protein. Flour contains very small amounts of sulfhydryl; the range reported according to most acceptable methods varies from 0.2 to 1.5 microequivalents sulfhydryl per g., depending on the method and the sample. Until recently, the major obstacle in studying the whole problem was the lack of an accurate method for sulfhydryl or there were limitations such as inadequate dispersions of gluten or flour to release all –SH groups, precipitation of the protein at the electrode, and other conditions of a particular method that gave results of doubtful value. This situation is not unique for flour but applies to many other complex biological materials. Bloksma (2) and Sokol, Mecham, and Pence (11,12) have discussed some of the problems in the measurement of thiol groups by amperometric titration.

Because of such problems in the quantitative measurement of sulfhydryl and the difficulty of obtaining adequate separations of constituent parts of flour, there is no unanimity concerning the location of –SH groups except that the water- and salt-soluble fractions of flour (albumins and globulins) contain sulfhydryl. There are conflicting results on the presence of –SH-containing compounds in the so-called gluten proteins and in the lipids.

This paper will discuss the measurement of sulfhydryl in the water extract of various milled fractions of a spring wheat, in the lipids separated from these fractions, and in some gliadin, albumin, and globulin preparations. A new technique, using PCMB to protect the –SH groups from oxidation during measurement, was employed to determine the total as well as the water-soluble –SH content of flour.

Materials and Methods

The method outlined by Kolthoff, Stricks, and Morren (4) using mercuric chloride as the titrant has given the most consistent results for flour. When gliadin or any lipid or other alcohol-soluble material is to be measured, the method of Benesch, Lardy, and Benesch (1) using silver nitrate is preferred.

Distribution of Sulfhydryl in Water Extract of Milled Fractions. Samples of a short patent flour, a first clear flour, a second clear flour, bran, and germ were taken at the same time from a spring wheat mix of 14.0% protein. Forty grams of the flours and 20 g. of the bran and the germ were shaken in a nitrogen atmosphere with 100 ml. of glass-distilled water and centrifuged. The supernatant liquid was decanted, recentrifuged, and a suitable aliquot (usually 10 ml.) titrated amperometrically with 0.001*M* mercuric chloride, according to the method of Kolthoff *et al.* (4). The titration was carried out in 60 ml. of a solution that was 0.05*M* to borax and 0.1*M* to potassium chloride.

Sulfhydryl of Lipids. The same mill fractions and, in addition, a soft wheat sample were shaken in an atmosphere of nitrogen with water-saturated n-butanol and centrifuged. The supernatant extract was decanted and recentrifuged after drying with anhydrous sodium sulfate. An aliquot of the extract was titrated amperometrically using a rotating platinum electrode and 0.001*N* silver nitrate, according to the method described by Benesch *et al.* (1). Nitrogen and phosphorus (8) in the lipid were also determined.

Sulfhydryl of Protein Fractions. Three albumin and three globulin preparations described by Pence and Elder (9) were measured by the argentometric amperometric titration.

Gliadin. Gliadin was isolated from a spring wheat patent flour and titrated in alcohol solution amperometrically by the method of Benesch *et al.* (1) using "tris" buffer. A commercial sample of gliadin secured from the Huron Milling Company also was tested.

Sulfhydryl of Gluten. Gluten recovered by the conventional washing process contains significant amounts of albumins and globulins, as has been emphasized by Pence *et al.* (10). Therefore, –SH results on washed gluten would be high as a result of the adsorbed albumins and globulins. In order to eliminate this possibility and to find out if the more insoluble, higher-molecular-weight proteins contained sulfhydryl, several techniques were tried. Kong, Mecham, and Pence (5) used a tryptic digest to solubilize the flour protein. Their amperometric titration values of dilute buffer extracts of untreated and trypsin-treated flour showed –SH groups probably to be absent from gluten.

In this laboratory, several experiments using trypsin were tried, but the results were not satisfactory. They varied widely, depending on the temperature of the digest, and were generally unreliable. Sokol *et al.* (11) showed, in further work, that maximum sulfhydryl values obtained for trypsin digests of flour were only about one-third as large as those of flour dispersions.

Some further preliminary tests were conducted on wet gluten washed out in the conventional manner from 10 g. of spring wheat patent flour (0.39% ash and 12.30% protein at 14.0% moisture). The gluten was dispersed in 8*M* urea and acetic acid under nitrogen and titrated amperometrically with 0.001*M* mercuric chloride. Lower values were obtained than those calculated as the difference between total sulfhydryl and water-soluble sulfhydryl of the flour from which the gluten was washed. The low values were probably the result of incomplete dispersion and of precipitation at the electrode. When the wet gluten was washed with 0.5*M* sodium chloride, the gluten still

contained about half of the total sulfhydryl.

Effect of Temperature. Another factor that has caused discrepancies in results is temperature. Sokol *et al.* (11) found that a rapid loss of sulfhydryl occurs in flour-buffer-urea dispersions when the dispersions are made at room temperature. Significantly higher results were obtained at 2°C. than at 25°C. A similar trend has been observed in this laboratory. The following experiment was designed to measure the effect of temperature on –SH determinations of a untreated spring wheat, straight-grade flour.

Five grams of a straight-grade flour were dispersed under nitrogen in 100 ml. 8*M* urea in a Stein Mill operating 5 seconds per minute for 10 minutes. The resulting solution was made 0.5*M* to potassium chloride and 0.05*M* to borax and titrated under nitrogen with 0.001*M* mercuric chloride. The temperature of the dispersion and the temperature of the titration were both maintained at just below 10°C. in one experiment and at room temperature (25°C.) in another test. Then the temperature of the dispersion was kept under 10°C. but the titration conducted at 25°C., and, finally, the temperature of the dispersion was maintained at room temperature but the titration conducted at just below 10°C.

Protection of –SH Groups from Oxidation during Measurement. *p*-Chloromercuribenzoate (PCMB) reacts specifically with –SH groups. Some observations by Dahle and Sullivan (3) indicated that, although PCMB reacts with free sulfhydryl groups, these sulfhydryl groups can still be measured by amperometric titration with mercuric chloride. Presumably, mercuric chloride displaces the PCMB from a –SH group, the former having a stronger affinity for the –SH group than the latter. This offers a useful technique for protecting –SH groups during measurement. This was checked by measuring the sulfhydryl of two glutathione (GSH) solutions, identical except for the presence or absence of PCMB.

Sixty milliliters of buffer (0.05*M* borax, 0.1*M* potassium chloride) were purged with nitrogen until very little oxygen was present, as indicated by deflection of the galvanometer from zero position; 2 ml. of a GSH solution were added and titrated, measuring 1.91 ml. 0.001*M* mercuric chloride. An identical procedure was followed using 60 ml. of buffer containing 5 micromoles PCMB; this measured 1.88 ml. of 0.001*M* mercuric chloride. The difference of the two values lies within the limits of accuracy of measurement. Although the GSH solution had been made to be 0.001*M*, it is likely that the molarity was slightly less since the GSH was not subjected to a preliminary drying before weighing.

Titrations of total sulfhydryl of flour were now attempted in the presence and absence of PCMB. The procedure involved sifting flour from a salt shaker into 8*M* urea previously purged with nitrogen, with constant swirling of the beaker to effect dispersion of the flour. The flour dispersion was made 0.05*M* to borax and 0.5*M* to potassium chloride and then titrated. The optimal amount of flour that could be titrated was found to be 5 g. dispersed into 200 ml. 8*M* urea.

Attention was now turned to a measurement of the water-soluble portion of flour sulfhydryl, where oxidation is more of a problem. The best procedure found was to drop 10 g. of flour in a single portion into 200 ml. of water (previously purged with nitrogen) in a 500-ml. cylinder, stopper the cylinder, and shake manually for 1 minute. The flour seemed completely dispersed after this treatment. The flour dispersion was centrifuged 10 minutes at 1,500 r.p.m. One hundred milliliters of the supernatant extract were pipetted and transferred to a beaker containing 48 g. of urea. To this were added 25 ml. of buffer (2.75*M* potassium chloride, 0.275*M* borax). The solution then was titrated.

Results

Distribution of Sulfhydryl in Water Extract of Milled Fractions. The results on the distribution of sulfhydryl in the water extract of milling separations from a spring wheat are shown below. The

Product	*–SH Content per g. Product* μeq.
Patent	0.12
First clear	0.17
Second clear	1.33
Bran	4.75
Germ	27.75

sulfhydryl in germ and bran is due to glutathione. No free glutathione can be found in flour except for low-grade or second clear flours containing significant amounts of germ and the scutellum layer.

Sulfhydryl of Lipids. Table I gives results on the sulfhydryl, nitrogen, and phosphorus of lipids extracted from a soft wheat and from milled products of a spring wheat. All lipid fractions gave negative results for sulfhydryl with the exception of the germ.

Starch free from gluten showed a negative test for sulfhydryl, as would be expected and has been shown previously by work in this laboratory and by other investigators (Sokol, Mecham, and Pence, 11).

All investigators are agreed that the soluble proteins contain –SH groups, but reports have varied concerning the presence of sulfhydryl

TABLE I

ANALYSES OF LIPIDS OF MILLED PRODUCTS[a] AND A SOFT WHEAT

	NITROGEN IN LIPID	PHOSPHORUS IN LIPID	MOLE-RATIO N:P	SULFHYDRYL IN LIPID
	%	%		%
Patent	0.63	0.97	1.2:1	0
First clear	0.50	0.76	1.4:1	0
Second clear	0.44	0.52	2:1	0
Bran	...	...	...	0
Germ	...	...	...	0.01
Soft wheat	0.70	0.70	1.9:1	0

[a] The milled fractions were obtained from a spring wheat mix of 14.0% protein.

in the more insoluble proteins.

Table II gives some figures for the –SH content of some albumin and globulin preparations. Both these fractions occur in the water extract of flour. Albumins and globulins are adsorbed to some degree on washed gluten.

TABLE II

SULFHYDRYL CONTENT OF ALBUMIN AND GLOBULIN FRACTIONS

	VARIOUS COMPONENTS	–SH PER GRAM PROTEIN
	%	μeq.
Albumins[a]		
Comanche		4.5
Pentad		6.1
Thatcher		6.6
Globulins[a]		
Germ	70 , 20 ,	6.1
Germ	20 , 68 , 11	6.1
Flour	44 , 46 , 7	6.6

[a] These samples were a few years old and there may have been some oxidation of –SH.

Sulfhydryl of Gluten Proteins. The gliadin preparation isolated in our laboratory from a spring wheat, patent flour contained 1.2 μeq. sulfhydryl per g. A commercial sample of gliadin showed 1.3 μeq. sulfhydryl per g. After this gliadin solution had stood for 62 hours, the –SH value dropped to 0.2 μeq. per g. As has been mentioned, wet gluten dispersed in 8*M* urea and acetic acid and measured amperometrically with mercuric chloride gave lower –SH values than those calculated as the differences between total flour sulfhydryl and water-soluble sulfhydryl. While the low results on gluten may be partly explained by precipitation at the electrode, other factors such as temperature affect the results of the –SH determination. Thus, a straight-grade flour gave the following figures with changes in the temperature of the dispersion and the temperature of titration:

Temperature of Dispersion	*Temperature of Titration*	*–SH per g. Flour* μeq.
<10°C.	<10°C.	0.42
room	room	0.23
<10°C.	room	0.23
room	<10°C.	0.26

The end point was not as sharp at low-temperature dispersion and low-temperature titration. The higher values obtained at lower temperature may be attributed to some labile compound that acts on sulfhydryl but at a lesser rate at lower temperature, or some configuration that is altered at relatively higher temperatures. It is further suggested by these data that the phenomenon is an irreversible one. Subjection of a dispersion to cold did not result in a higher value after the dispersion had once been at room temperature.

It was apparent that, since starch and lipids of flour contain no –SH groups and since gluten cannot be separated without some adhering globulins and albumins, a measurement of total flour sulfhydryl minus water and/or salt-soluble sulfhydryl would have to suffice as an index of the –SH content of the gluten proteins. Some investigators have reported that gluten proteins contained no sulfhydryl, perhaps because of the great difficulty in adequately dispersing the sample and the lability of the –SH groups.

Protection of –SH Groups from Oxidation during Measurement. As has been noted, PCMB can be used to protect –SH groups during measurement. Matsumoto and Shimoda (7) had observed that PCMB did not lower the titration value obtained by the mercurimetric titration. Results differed from those obtained with silver nitrate in the presence of PCMB. In a more recent paper, Matsumoto and Hlynka (6) reported on the –SH content of various flour fractions and, further, that PCMB decreased the –SH content of water-soluble and acid-soluble fractions of dough by 76 and 86%, respectively, when measured amperometrically with silver nitrate.

Five grams of untreated straight-grade spring wheat flour dispersed in urea measured 2.4 ml. 0.001*M* mercuric chloride to give a value of 0.56 μeq. sulfhydryl per g. of flour (dry basis). An identical procedure using 200 ml. of 8*M* urea containing 5 micromoles PCMB measured 0.62 μeq. sulfhydryl per g. of flour (dry basis). The water-soluble extract, which is more vulnerable to oxidation, measured 0.35 ml. 0.001*M* mercuric chloride to give a value of 0.08 μeq. sulfhydryl per g. of flour (dry basis). An identical procedure using 200 ml. of water containing 10 micromoles PCMB measured 1.2 ml. 0.001*M* mercuric

chloride or 0.28 μeq. sulfhydryl per g. A tabulation of these values follows:

–SH	–SH per g. Flour (dry basis)	
	No PCMB	*With PCMB*
	μeq.	μeq.
Total	0.56	0.62
Water-soluble	0.08	0.28
Difference	0.48	0.34

Since it is not possible to maintain a completely air-free environment in the procedure for the determination of water-soluble sulfhydryl, it is likely that a loss is effected through air oxidation. The higher the values obtained, the shorter the time between dispersion of flour and measurement of the supernatant liquid. Since sulfhydryl in the presence of PCMB ought to be protected from loss by other agents such as air or oxygen, the PCMB values might be considered as the true figures. The difference of total and water-soluble sulfhydryl might be regarded as representing the nonwater-soluble sulfhydryl in the procedure using PCMB.

Using the PCMB values as a reference, it might be said that $((0.62 - 0.56)/0.62) \times 100$, or 9.7%, of total sulfhydryl is lost in the procedure deleting PCMB, and $((0.28 - 0.08)/0.28) \times 100$, or 71%, of water-soluble sulfhydryl is lost in the procedure involving its measurement. Assuming air oxidation to have a negligible effect in the measurement of *total* sulfhydryl (either in the presence or absence of PCMB), it must be said that the loss of water-soluble sulfhydryl is much greater when PCMB is not present. The difference in *water-soluble sulfhydryl* measured with and without PCMB is 0.20 (0.28 – 0.08), whereas the difference in total sulfhydryl with and without PCMB is 0.06 (0.62 – 0.56). Thus, there is about four times as much sulfhydryl lost in the procedure for water-soluble as for total sulfhydryl. We believe this loss is due to oxidation of sulfhydryl by thioctic acid monoxide (3). There is probably a more intimate mixture of sulfhydryl and –SH-reacting factors in a water extract of flour than in a total flour dispersion where adsorption could inhibit their free movement. The reason for this loss will be explained in a later paper.

Generally, with all precautions taken, such as minimizing or eliminating oxidation by blowing nitrogen through solutions, by keeping temperatures low, and by protecting available –SH groups by PCMB, the results shown in this paper average somewhat lower than many figures reported by other investigators.

With the use of the best techniques currently available, –SH groups of flour appear to be about equally distributed between the water-soluble proteins and the gluten proteins, with none present in the lipid and none, of course, present in the starch.

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THE OXIDATION OF WHEAT FLOUR

II. Effect of Sulfhydryl-Blocking Agents[1]

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ABSTRACT

Certain sulfhydryl-blocking reagents not only shorten the mixing time of a flour, but also, when used in sufficient amounts to combine with –SH groups, eliminate the beneficial effect of maturing agents such as potassium bromate and potassium iodate. Extensigraph and baking data show that both N-ethylmaleimide and *p*-chloromecuribenzoate increase extensibility and decrease resistance to extension as compared to the control; these effects are not changed when bromate or iodate is added after the addition of the –SH-blocking agents. This gives further support to the involvement of –SH groups in flour maturing.

It is possible that intramolecular S–S bonds are changed to intermolecular S–S cross linkages by means of small amounts of –SH groups. Whereas the disulfide bonds may be primarily responsible for toughness and strength of a dough, there are undoubtedly weak linkages, such as thiolester, amide or hydrogen bonds, that require less energy to break and re-form.

The action of the optimum amount of a maturing agent on flour produces a dough with better machining characteristics and an improved baked product. This oxidation involves the sulfhydryl groups, but the relative importance of other groups and the manner of cross-linking of the protein chains are not known. The general mechanism of each of the improvers is apparently different, as reflected in the rheological properties of the doughs.

Mecham (4) and Mecham, Sokol, and Pence (5) have shown that certain of the –SH reagents, such as N-ethylmaleimide (NEMI) and *p*-chloromercuribenzoate (PCMB), affect the mixing time of a flour, shortening the time to maximum resistance and increasing the rate of breakdown. One might expect the opposite since a weakening of the curve is characteristic of the behavior of such compounds as glutathione, cysteine, and sulfite that act on the S–S bond.

Goldstein (3) showed, by means of extensigrams after a 135-minute rest period, that small amounts of ascorbic acid and potassium bromate improved the properties of dough considerably and that, when all available –SH groups were blocked with PCMB, addition of ascorbic acid and potassium bromate was without effect. This evidence supplies additional proof that improvers act on –SH groups. Goldstein pointed

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out, however, that, because of the very small amounts of free –SH groups which gluten contains, it is unlikely that two –SH groups would be in close enough proximity to be oxidized to an S–S bond. He postulated that improvement can be more adequately explained by the action of the maturing agent in hindering an exchange between –SH and S–S groups. More recently, Frater, Hird, Moss, and Yates (2) presented some interesting data on the effect of iodate, N-ethylmaleimide, and cysteine on the rheological properties of dough. They think it likely that, at any given protein content of flour, the rheological properties of dough are directly related to the number of intermolecular disulfide bonds and the rate at which they can interchange with thiol groups. Since the S–S groups (as cystine) are present in five to ten times the amount of –SH groups, the interchange of –SH with S–S would seem to be one logical explanation. In order to investigate this subject further, some of Goldstein's experiments were repeated and extended as a basis for further elucidation of the reaction mechanism of flour oxidation.

Materials and Methods

An untreated spring wheat, straight-grade flour of 0.43% ash, 12.50% protein, and 185 maltose value was used in these experiments.

The sodium salt of PCMB (Sigma Chemical Co., St. Louis, Mo.) was made up 0.3 g. to 100 ml. of water; 0.2 ml. of 6*N* sodium hydroxide was added to obtain complete solution. NEMI was obtained from Eastman Kodak Co. and, without further purification, made up at the same concentration as the PCMB.

Baking Tests with PCMB. A series of baking tests was performed to measure the effect of PCMB, bromate, and iodate. The flour was malted with 0.5% malt. A straight-dough formula was used, as follows: 300 g. flour at 14.0% moisture, 62.0% absorption, 2% salt, 4% sugar, 3% shortening, and 2% yeast. Doughs were mixed on a Hobart mixer to their optimum development and machined and baked in the conventional manner. When PCMB was used, the mixing was interrupted for 2 minutes to allow time to react before iodate or bromate was added. Since the dough quality and volume of the bread reflected sufficiently the action of the added reagents, these two factors are reported for breads made from the untreated flour and from the same flour to which PCMB was added in varying amounts. Iodate and/or bromate were also added in various amounts, and different levels of iodate or bromate were superimposed after the PCMB treatment.

Farinograph and Extensigraph Data. Farinograms were made by the constant-dough (480 g.) method (1). Extensigrams were obtained

as follows: a farinogram of the flour, reagent, and water was first obtained to determine the correct absorption. The extensigraph doughs were all given a 5.5-minute total mixing time, as found optimum from the farinogram of the control flour. All doughs were nonyeasted and contained 2% salt based on the flour weight. All the absorption water, including the small amount (20 to 40 ml.) used to dissolve the –SH reagent, was added to the flour in a 300-g. mixing bowl. The dough was mixed 1 minute, followed by a 12-minute rest period. The remaining 5.0 ml. of water or solution containing the improver, if any, were added and the dough remixed for 4.5 minutes.

After the dough was removed from the farinograph bowl, two 150-g. portions were scaled, each rounded 20 times, molded into dough cylinders, and immediately placed in the fermentation cabinets at 30°C. The doughs were allowed to relax 45 minutes and stretched. This procedure was repeated at 90, 135, and 180 minutes.

The control flour was measured in a similar manner and the results averaged. Results are expressed as resistance, extensibility, and area at 45, 90, 135, and 180 minutes.

Results

Mixing Curves. Farinograms of the flour with no treatment and with NEMI (15 mg.%), PCMB (30 mg.%), and iodate (3 mg.%) are shown in Fig. 1.

Small amounts of NEMI and iodoacetamide (about equivalent amounts to combine with sulfhydryl) weaken a mixing curve (4). PCMB and iodate behave in a similar manner, as Fig. 1 shows, but, as is well known, bromate does not affect the curve.

Response of Iodate and Bromate to PCMB-Treated Flours. The baking results on flour to which PCMB, iodate, and bromate were added are summarized in Table I.

Table I shows that PCMB improves the volume of an untreated flour below the critical level of binding of –SH groups. Iodate and bromate give the usual beneficial effect. When most, or all, of the sulfhydryl groups are bound (with this particular flour, this occurs at slightly over 0.025% PCMB), the dough becomes stringy and extensible, the volume decreases, and iodate or bromate superimposed on the PCMB-treated flour shows no beneficial effect.

Extensigraph Data. Figure 2 and Table II give results for the control flour and the same flour to which was added 5 mg.% of potassium bromate and 1 mg.% of potassium iodate.

The usual increase in resistance and decrease in extensibility compared to the control are observed. The effect of bromate and iodate,

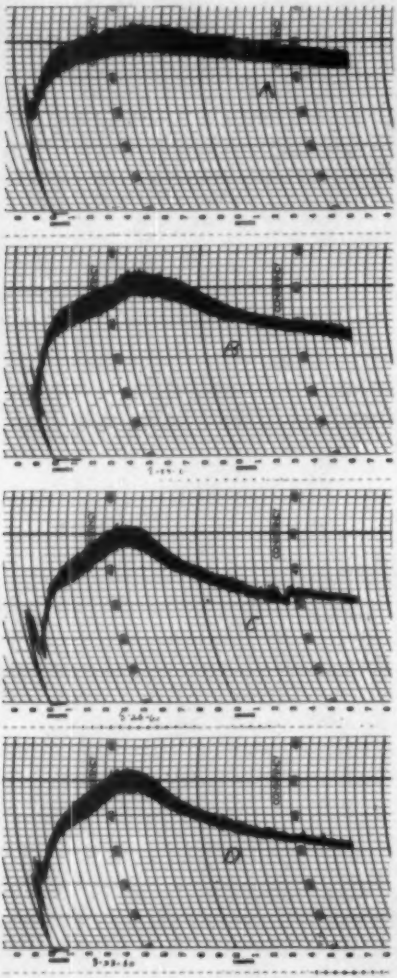

Fig. 1. Farinograms showing effect of iodate, NEMI, and PCMB on mixing characteristics. All curves based on 480-g. dough at 500 B.u. A, control (untreated flour), 61.8% absorption at 14.0% moisture; B, control plus 3 mg.% potassium iodate, 63% absorption at 14.0% moisture; C, control plus 15 mg.% N-ethylmaleimide, 63.3% absorption at 14.0% moisture; D, control plus 30 mg.% *p*-chloromercuribenzoate, 64.9% absorption.

as measured on the extensigraph, is not as pronounced in a flour-water-salt dough as in a dough containing yeast. No yeast was used in

TABLE I

EFFECT OF PCMB AND SUBSEQUENT OXIDIZING TREATMENT ON A PATENT FLOUR

	PCMB[a]	IODATE[b]	BROMATE[c]	DOUGH QUALITY	VOLUME
	%	ppm	ppm		
Control	0.0	0.0	0.0	Elastic	100
	0.0	5.0	0.0	Slightly tough	104
	0.0	7.5	0.0	Slightly tough	107
	0.0	10.0	0.0	Elastic, slightly tough	112
	0.0	12.5	0.0	Tough	114
	0.0	15.0	0.0	Tough	115
	0.0	25.0	0.0	Very tough	116
	0.0	0.0	10.0	Slightly tough	113
	0.0	0.0	15.0	Slightly tough	121
	0.0	0.0	20.0	Tough, slightly short	119
	0.0	0.0	25.0	Tough, short	120
	0.0	0.0	50.0	Very tough	115
	0.01	0.0	0.0	Slightly short, slightly soft	108
	0.02	0.0	0.0	Slightly soft, extensible	113
	0.03	0.0	0.0	Soft, extensible	89
	0.04	0.0	0.0	Stringy, very soft	78
	0.0175	0.0	20.0	Slightly soft, extensible	116
	0.0200	0.0	20.0	Slightly soft, extensible	116
	0.0250	0.0	20.0	Slightly soft, extensible	100
	0.0275	0.0	20.0	Soft, extensible	88
	0.0300	0.0	20.0	Short, stringy, soft	81
	0.0300	0.0	50.0	Short, stringy, soft	92
	0.0330	0.0	20.0	Short, stringy, very soft	70
	0.0450	0.0	20.0	Short, stringy, very soft	53
	0.02	10.0	0.0	Slightly tough	101
	0.03	25.0	0.0	Soft, extensible, stringy	83

[a] A level above 0.025% PCMB was needed to exceed the optimum level as reflected in loaf volume. All PCMB doughs were exceedingly extensible; those with the high amounts of PCMB (over 0.03%) were stringy.

[b] This flour had a higher-than-normal tolerance to iodate. Iodated doughs were all on the tough side until they came to the molder; then they started to mellow out. With this flour, 10 p.p.m. iodate gave the best loaf.

[c] The optimum bromate response was given at 10 p.p.m. Amounts of 20 and 25 p.p.m. gave doughs that were tough. Dough characteristics and loaves below standard were produced by 25 p.p.m. iodate and 50 p.p.m. bromate.

any of the extensigraph experiments in order to avoid any further complications in interpretation.

The same control flour was tested on the extensigraph with 10 and 15 mg.% of N-ethylmaleimide (NEMI). There is a sharp decrease in R and increase in E values; the higher amount of NEMI caused the greater change, as shown in Table II. This –SH reagent, like PCMB, increases extensibility rather than decreasing it as many maturing agents do. Three samples of the control flour were each treated with 15 mg.% of NEMI. One sample was then treated, after a rest period, with 5 mg.% potassium bromate and the other with 1 mg.% potassium iodate. Results are shown in Table II and Fig. 2.

TABLE II

DEPRESSION OF RESPONSE TO IODATE AND BROMATE IN DOUGHS CONTAINING N-ETHYLMALEIMIDE (NEMI)

	Treatment																				
	No Treatment			5 mg.% $KBrO_3$			1 mg.% KIO_3			10 mg.% NEMI			15 mg.% NEMI			15 mg.% NEMI +5 mg.% $KBrO_3$			15 mg.% NEMI +1 mg.% KIO_3		
Time	R[a]	E[a]	A[a]	R	E	A	R	E	A	R	E	A	R	E	A	R	E	A	R	E	A
minutes																					
45	450	220	124	495	203	127	540	202	137	215	209	58	125	257	39	160	230	48	150	208	40
90	710	200	177	780	183	181	810	150	150	275	187	68	180	215	47	200	210	56	170	225	46
135	830	151	155	885	135	146	940	133	151	290	194	73	160	250	45	200	205	51	155	265	49
180	930	125	144	970	105	120	1000	114	136	290	185	67	170	235	44	200	230	55	165	252	46

[a] R = resistance; E = extensibility; A = area.

TABLE III

EFFECT OF PARA-CHLOROMERCURIBENZOATE (PCMB) ON DOUGH MOBILITY AS MEASURED BY THE EXTENSIGRAPH

	Increments of PCMB (in mg. %)																				
	0			10			15			20			25			30			35		
Time	R[a]	E[a]	A[a]	R	E	A	R	E	A	R	E	A	R	E	A	R	E	A	R	E	A
minutes																					
45	450	220	124	430	218	119	380	203	100	310	190	79	190	225	62	100	235	28	110	200	25
90	710	200	177	690	172	150	650	165	139	590	150	114	430	140	83	150	230	40	130	176	27
135	830	151	155	870	145	156	740	138	128	800	115	112	625	115	94	195	205	49	130	178	25
180	930	125	144	990	120	139	890	122	134	845	108	690	690	106	92	230	185	54	150	168	31

[a] R = resistance; E = extensibility; A = area.

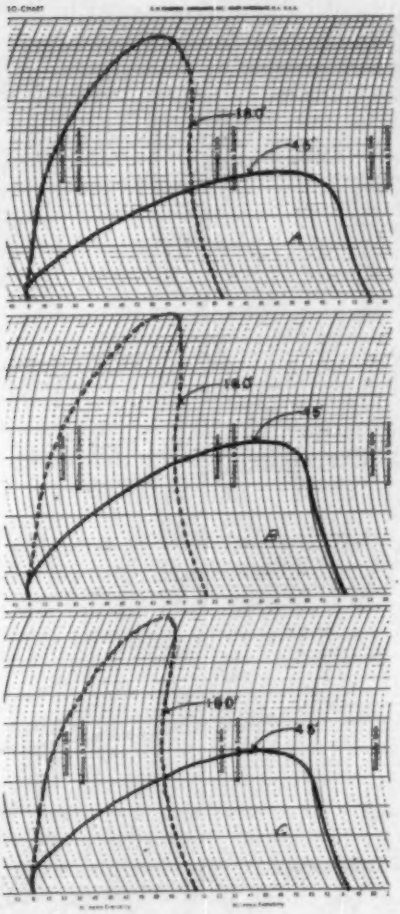

Fig. 2, A–C. Extensigraph data on the effect of iodate and bromate after blocking of –SH groups. A, untreated flour; B, 1 mg.% potassium iodate; C, 5 mg.% potassium bromate.

The maturing effect of both bromate and iodate was eliminated when those treatments were superimposed on NEMI-treated flour, although the bromate showed a slight increase in R values as compared to the NEMI control.

PCMB is another reagent considered specific for the sulfhydryl groups. Table III illustrates the effect of increments of the sodium salt of PCMB, from 10 to 35 mg.%, on extensigraph curves.

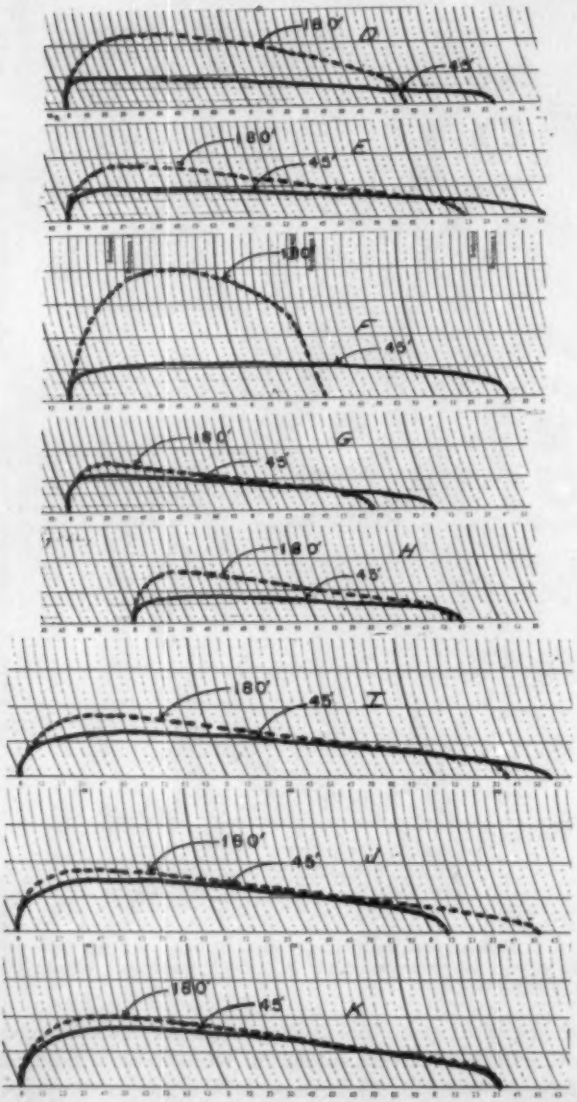

Fig. 2, D–K. Extensigraph data on the effect of iodate and bromate after blocking of –SH groups. D, 30 mg.% PCMB; E, 30 mg.% PCMB plus 1 mg.% potassium iodate; F, 30 mg.% PCMB plus 5 mg.% potassium bromate; G, 35 mg.% PCMB; H, 35 mg.% PCMB plus 5 mg.% potassium bromate; I, 15 mg.% NEMI; J, 15 mg.% NEMI plus 1 mg.% potassium iodate; K, 15 mg.% NEMI plus 5 mg.% potassium bromate.

TABLE IV

DECREASE IN BROMATE AND IODATE RESPONSE IN DOUGHS WITH INCREASING AMOUNTS OF PARA-CHLOROMERCURIBENZOATE (PCMB)

PCMB	TIME	TREATMENT[a]								
		0			3 mg.% $KBrO_3$			1 mg.% KIO_3		
		R	E	A	R	E	A	R	E	A
mg.%	*minutes*									
0	45	450	220	124	495	203	127	540	202	137
0	90	710	200	177	780	183	181	810	150	150
0	135	830	151	155	885	135	146	940	133	151
0	180	930	125	144	970	105	120	1000	114	136
25	45	190	225	62	275	200	77	190	219	58
25	90	430	140	83	525	135	95	333	177	78
25	135	625	115	94	730	103	91	380	137	70
25	180	690	106	92	790	97	96	380	140	74
30	45	100	235	28	115	240	39	95	262	30
30	90	150	230	40	265	175	65	150	245	42
30	135	195	205	49	330	142	66	160	220	43
30	180	230	185	53	400	140	73	175	218	44
35	45	110	200	25	80	180	17			
35	90	130	176	27	130	163	26			
35	135	130	178	25	160	176	33			
35	180	150	168	31	145	165	30			

[a] R = resistance; E = extensibility; A = area.

With higher levels of PCMB, the resistance decreases and the extensibility increases. The dough becomes soft and stringy. When bromate or iodate was added to the dough after addition of PCMB presumably sufficient to combine with all the –SH groups, there was no maturing effect. It is noteworthy, however, that, whereas 30 mg.% PCMB was sufficient to block the iodate reaction, 35 mg.% PCMB was required to block the bromate effect completely. Results are shown in Table IV and Fig. 2.

Discussion

Specific –SH reagents, such as NEMI and PCMB, not only affect the mixing time of flour but also increase the extensibility and decrease the resistance to extension; whereas common maturing agents produce a reverse effect. At first thought, it is puzzling that certain sulfhydryl-blocking agents weaken the mixing curve in a manner

analogous to that of –SH-containing compounds such as glutathione and cysteine. The rate to maximum and the drop after maximum sharply increase with both types of compounds. The –SH-containing compounds in sufficient amount break existing disulfide cross-links, resulting in a soft, sticky dough and a weak curve. Blocking agents, such as NEMI and mercury compounds, on the other hand, combine with the free –SH groups of the flour proteins, preventing the interchange with the S–S groups and the formation of new cross-links. This possibility, as well as others, has been discussed by Mecham (4) in relation to mixing behavior.

Specific –SH reagents increase extensibility and decrease resistance to extension according to the state of oxidation of dough during mixing and the consequent availability of –SH groups. Extensigraph and baking data confirm and extend the experiments of Goldstein (3), which showed that when –SH groups are blocked, maturing agents do not exert their beneficial effect. It was observed, however, that the amounts of NEMI and PCMB that stop the iodate reaction are not sufficient to inhibit completely the bromate effect. A level of NEMI and PCMB somewhat higher than the amount calculated to react with the total sulfhydryl is required to suppress completely the bromate response of the flour. This may mean that the –SH determination is too low or that the stoichiometric equivalents of NEMI or PCMB added would not react with all the –SH groups of a given flour within the duration of the experiment, even though complete reaction might be achieved under different conditions or over a longer period of time. It is also probable that the number of –SH groups available for titration after denaturation with urea is not the same as available in an undenatured dough.

The total sulfhydryl of the flour used in these experiments measured 0.41 micromoles per g. This amount would be equivalent to 5 mg.% NEMI or 16 mg.% PCMB. The total sulfhydryl of the flour was determined by amperometric titration with mercuric chloride. An equivalent amount of NEMI did not titrate all the sulfhydryl under the same conditions, although it appeared that about 75% of the total sulfhydryl could be titrated. Thus, it would appear that the amounts of some blocking agents required to bind the –SH groups of a dough may not reflect an accurate measure of sulfhydryl, since accessibility and reactivity of these groups to various blocking agents vary. Moreover, the extent of the response to blocking agents and improvers depends on the mixing and mechanical treatment given a dough.

When the usual maturing agents are employed, it would seem probable that some intramolecular S–S bonds are changed to inter-

molecular S–S cross-linkages through the mediation of very small amounts of thiol groups. It is presumed that the intermolecular disulfide bridges confer toughness and greater resistance to extension to the dough. When a dough stands, its flow properties increase with time. And, when energy is applied through work such as mixing, rounding, or molding, more opportunity is given for exchange of –SH and S–S groups. It is highly probable that many of the cross-links conferring beneficial elastic properties on dough are not covalent disulfide bonds, but weaker linkages such as thiolester or hydrogen bonds. The large number of amide side chains of the flour proteins would be expected to form hydrogen bonds that could be easily broken and re-formed. More intensive study is needed to determine the nature and energy of these bonds.

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NOTE ON A METHOD OF DETERMINING THE DEGREE OF MILLING OF WHOLE MILLED RICE[1]

Joseph T. Hogan and Harold J. Deobald

In conventional rice milling practice, the degree of bran removal is estimated by visual observation. A more reliable and consistent means of measurement is desirable, however, in order to obtain quantitative results in rice milling research. Commercial rice bran consists of the outside layers of the rice kernel removed during milling and is composed of the pericarp, testa, perisperm, most of the aleurone layer, some starchy endosperm, and the whole of the embryo. The protein content, fat or oil, and soluble carbohydrates of the bran are derived from the inner aleurone cells and the embryo; whereas the starch content is derived from the endosperm cells which adhere to

[1]Manuscript received May 9, 1960. Contribution from Southern Regional Research Laboratory, one of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

the outer bran layers and are removed in the milling (3).

Autrey and others (2) demonstrated in 322 tests of Zenith (medium-grain) and Rexark (long-grain) rices that the amount of fat extracted from whole milled rice was in linear relationship to the amount of bran removed up to about 6% of the original rice. They further showed that, for a given variety, the relationship between the percentage of bran removed and the percentage of fat remaining on the milled rice is constant from year to year. These studies indicate that reliable and consistent results can be obtained by using the extractable fats of the whole milled rice as a measure of the degree of milling. It serves as an approximate measure of the amount of inner bran (the aleurone layer) remaining on the milled rice.

The purpose of this communication is to report the details of a procedure for determining the extractable fats of whole milled rice. This procedure was used in the evaluation of some 600 samples of rice of different varieties and geographical origin. It varies in manipulative technique from that of Autrey *et al.* (2) and employs a petroleum solvent rather than diethyl ether. As pointed out by Shearer and Carson (4), petroleum solvents, as fat extractants, are preferable to anhydrous ether in many respects. Technical grades require no preparation before use; they have higher flash points, are not subject to oxidation, are not hygroscopic, and cost less.

Methods

Approximately 20 g. of a well-mixed sample of whole milled rice are weighed accurately into a 125-ml. flat-bottomed boiling flask. Twenty-five milliliters of petroleum ether, boiling range 35°–38°C. (AOCS (1) specification H2-41) are added to the flask containing the rice. The reflux apparatus, consisting of the 125-ml. flask and a West condenser, with standard-taper glass joints, is assembled, and the solvent is heated to boiling on a hot plate and refluxed for 25 minutes. After refluxing, the solvent is decanted from the flask (taking care to retain the rice in the flask) onto a folded filter (Whatman No. 12) held in a funnel placed in the mouth of a tared 100-ml. flat-bottomed extraction flask. Fifteen milliliters of petroleum ether are then added to the flask containing the rice. The solvent is allowed to come to a boil and decanted immediately onto the same filter previously used, again with care to retain the rice in the flask. The latter step, i.e. addition of 15 ml. of petroleum ether to the extraction flask, etc., is repeated, except that the rice and solvent are completely transferred to the filter. When the solvent flow ceases, the solvent is evaporated from the tared flask on a steam bath until no odor of solvent re-

mains, and the flask is placed in a forced-draft oven for 30 minutes at 101–104°C. The flask is removed from the oven, cooled in a desiccator, and weighed. The percentage of fats extracted is calculated on the basis of the dry weight of milled rice used.

Results

As an illustration of the application of the method, four samples of Century Patna 231 variety, foundation seed stock grown at the Rice Pasture Experiment Station, Beaumont, Texas, were each subjected to a different degree of milling with standard laboratory milling equipment (5,6,7,8). The whole milled rices were evaluated in duplicate by the above procedure. Table I presents the details of milling and the extractable fats of the whole milled rices.

TABLE I

THE EXTRACTABLE FATS OF WHOLE MILLED RICE AS RELATED TO THE DEGREE OF MILLING

SAMPLE	DESCRIPTION OF MILLING	EXTRACTABLE FATS
		%
1	Milled for 30 seconds without weight	0.60
2	Same as for sample 1	0.62
3	Milled for 30 seconds with 2-lb. weight	0.47
4	Same as for sample 3	0.44
5	Milled for 30 seconds with 2-lb. weight plus an additional 30 seconds without weight	0.39
6		0.37
7	Milled for 30 seconds with 2-lb. weight plus 1 g. of Carbotex[a]; plus an additional milling for 30 seconds without weight	0.32
8	Same as for sample 7	0.29

[a] Carbotex, a natural pulverized limestone.

Acknowledgment

The authors wish to thank John V. Halick, Rice Pasture Experiment Station, Beaumont, Texas, for supplying the milled rices used in this study.

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BUBBLE MECHANICS IN THICK FOAMS AND THEIR EFFECTS ON CAKE QUALITY[1]

AVROM R. HANDLEMAN, JAMES F. CONN, AND JOHN W. LYONS

ABSTRACT

Characteristics of finished cakes are compared with those predicted by a theoretical treatment of properties of the batter which would affect the growth and movement of bubbles. Surface tensions, viscosities, and yield values were measured in several white and yellow cake batters, and the rates of bubble-to-bubble gas diffusion were observed in sequential photomicrographs of a single field of each batter. It was observed that, in batters prepared with unemulsified shortening, bubble-to-bubble diffusion was extremely slow, and leavening gas was evolved into only a small percentage of the bubbles. As predicted by the mathematics, when a given amount of gas is evolved into a small number of bubbles, a large proportion attain critical bouyancy and rise. These cakes had lower volumes, indicating that a significant proportion of the gas was lost, presumably by this mechanism. Where diffusion was more rapid and involved larger numbers of bubbles, volumes were higher. In a case where the yield and viscosity values were high, even the tendency to layer (larger cells to accumulate nearer the surface) was minimized.

It is generally accepted that the cells of a cake originate as bubbles in the batter. Pneumatic support provided by the bubbles maintains the structure of a cake until mechanical strength is developed in the nongaseous phases. Hence, the strength, texture, volume, shape, and grain of cakes are determined by size distribution, inflation, movement, film permeability, and stability of bubbles, along with mechanical properties developed in the setting of the batter.

Since the mathematics of bubble formation, growth, and movement have been developed fairly well, it was thought worth while to determine where they provide explanations for some of the observations made in practical cake-batter systems. Knowledge of this type should reduce the experimentation required in product development.

In this study, viscosities and yield values of cake batters have been measured; two types of interfacial tensions have been measured; accompanying bubble distribution and growth characteristics have been observed in the cold batter; and cakes resulting from these batters have been evaluated. Batters and cakes studied were all prepared with one base formulation. Whole egg, egg white, and egg white plus 0.1% lecithin (dry mix basis) were used with three different commercial shortenings, which varied primarily in type of emulsification. These formulation variations were chosen to provide a range in the

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measured and observed properties, so that trends could be recognized and compared with those predicted by theory.

Materials and Methods

Batter Preparation. Large batches of dry mix were prepared by commercial procedures with pilot-plant equipment, according to the formulation given in Table I. Each of three test shortenings was

TABLE I

FORMULATION OF DRY MIXES USED TO PREPARE EXPERIMENTAL BATTERS

INGREDIENT	TYPE	PERCENT OF DRY MIX
Flour	Commercial cake – 9.3% moisture, 8.7% protein (14% moisture basis), 0.36% ash, AACC viscosity 37, pH 4.85	36.2
Sugar	Baker's Special fine granulated	45.3
Shortening	Varied; types described below	12.0
Milk	High-heat, spray-dried, nonfat dry	4.0
Salt	Reagent grade, unfilled	0.8
Sodium bicarbonate	Granular (about 5% retained on 100-mesh and about 5% through 270-mesh)	0.7
Sodium Acid Pyrophosphate	Slow,[a] food grade	0.7
Monocalcium Phosphate Monohydrate	Food grade	0.3

[a] Trademark RD-1.

used. Batters were prepared by the following three-step mixing procedure in a Hobart N-50 mixer with a 4-qt. stainless-steel bowl and paddle.

1. Dry mix, 1,000 g., was blended with 130 g. tapwater for 15 seconds at speed 1 and beaten 2 minutes at speed 2; bowl scraped down.
2. Water, 100 g., and half the egg were added and blended 15 seconds at speed 1 and beaten 1 minute at speed 2; bowl scraped down.
3. The remaining egg and 170 g. of water were added and blended 15 seconds at speed 1 and beaten 1 minute at speed 2.

Cakes. Cakes were baked from one-fourth of each batter in 8-in. aluminum cake pans at 350°F. for 35 minutes.

Formulation Variations. The three commercial shortenings used were: 1) a hydrogenated vegetable shortening (unemulsified), 2) a normal high-ratio shortening containing mono- and diglycerides and lechithin, and 3) a high-aerating shortening containing mono- and diglycerides and probably a lactylated monoglyceride emulsifier.

With each of these shortenings, three test batters were studied. These batters varied in that they contained, in addition to water,

1) 120 g. egg white; 2) 120 g. egg white and 72 g. egg yolk; or 3) 120 g. egg white and 1 g. lecithin.

The lecithin was added as a 20% hydrated slurry at the beginning of the first stage of mixing. The material was a commercial drug product obtained from soybeans. This design permits detection of effects and interactions of shortening emulsification, egg yolk, and lecithin, introduced into the aqueous phase.

Formulation Fractions for Interfacial Tension Measurement. Three aqueous systems were prepared for interfacial tension measurements against each of the shortenings. The components of these aqueous systems were present in the same proportions used in the complete batters. Systems prepared were 1) sugar, water, and egg white; 2) sugar, water, egg white, and egg yolk; 3) sugar, water, egg white, and lecithin.

Interfacial Tension Measurements. The air or gas-liquid interfacial tension acting on a single bubble in cake batter was calculated from the maximum pressure reached in blowing a bubble with a capillary of known inside diameter submerged in the batter at a known depth. After correcting for depth of submergence, γ can be calculated from equation 5. The radius of the capillary is the minimum radius of curvature which a bubble at the tip can attain, and, therefore, is the radius at maximum pressure. This radius was calculated from the maximum bubble pressure attained with water and literature value for the surface tension of water at its air interface. Measurements were made at room temperature with each experimental batter.

Interfacial tensions were measured between the shortenings and some components of the aqueous phase of the batters by a pendant drop technique (1) with measurements made on photomicrographs of the drop and capillary.

Viscosity Measurements. Apparent viscous coefficients at varying shear rates were determined for each batter with a MacMichael Viscometer. Results showed a significant increase in apparent viscosity as shear rate decreased.

Yield Value Measurements. Yield value was calculated from the residual force on a metal plate vertically suspended in a test batter after displacement of the plate and 5-minute relaxation of the batter.

Bubble Distribution and Growth. Carlin (3) photographed bubbles in cake batters to show effects of shortening emulsification. In the present study, however, sequential photomicrographs of a single field were taken over a period of 90 minutes with each test batter. Slides were prepared by gently pressing a small drop of batter between a slide and cover glass and then sealing the cover glass boundary with stopcock grease to reduce drying of the batter. Only transmitted light

was used, although it was observed that reflected light sharpened boundaries between aqueous and fat phases. Microscope magnification was 60× and this was approximately tripled in the photographs.

Cake Volume and Conformation. Cakes were measured according to the method developed by the AACC committee on cake flour testing. The height of a center cross-section was measured at the center and 60 mm. in each direction from the center. Height measurements were reported in the following order: side, center, side.

The radius was also measured and the volume calculated from this and the average height. Specific volume was the calculated volume in cc. divided by the batter weight in g.

Finished Cake Photographs. Finished cake photographs provide an excellent record of many subjective characteristics of baked products, and a standardized method has been adopted in this laboratory and was used in this study. Cakes were photographed, one at a time, with half a layer standing on its cross-section and a thin section of the cross-section placed in front. This arrangement was adopted to show the most crust, shape, and texture characteristics.

The following precautions were taken to assure uniform photographic conditions: 1) The two lights and Crown Graphic camera were mounted in fixed positions with fixed settings, and the subject was positioned with a template; 2) the Panatomic-X sheet film was developed by a set, reproducible procedure, which limited variations due to developer age, development time, etc.

Negatives were sufficiently uniform to permit compositing for useful comparisons. Further, the procedure, once established, is sufficiently simple and rapid to be used routinely with baking studies.

Results and Discussion

Bubble Mechanics (1,2). For a single, submerged bubble in an ideal thick-walled foam:

$$F_1 = \pi\ r^2 p, \quad (1)$$

$$F_2 = 2\pi\ r\gamma, \quad (2)$$

$$F_3 = 4/3\pi\ r^3 g(\rho_2 - \rho_1), \text{ and} \quad (3)$$

$$F_4 = 6\pi\ r\eta\sigma. \quad (4)$$

In these expressions:

F_1 = force tending to expand the bubble;

F_2 = force tending to contract the bubble;

F_3 = bouyant force tending to make the bubble rise;

F_4 = frictional force exerted against a spherical body moving in a viscous fluid (Stokes' law);

r = radius of bubble;

p = pressure of gas in bubble in excess of hydrostatic;
γ = surface tension at the gas-liquid interface;
g = gravitational constant;
ρ_1 = density of gas in bubble;
ρ_2 = density of liquid in foam;
η = Newtonian viscosity;
σ = rate at which the sphere moves in the fluid.

Equating the expressions for F_1 and F_2 and solving for p, it can be shown that, for a bubble in equilibrium (1),

$$p = 2\gamma/r \tag{5}$$

From equation 5, it is apparent that, as bubbles become extremely small, their internal pressures become exorbitantly large, so that spontaneous nucleation of bubbles in a continuous fluid is extremely unlikely. This difficulty is overcome in cake batters by mechanically entraining and subdividing bubbles of air and leavening gas during mixing. These bubbles then provide the majority of sites for collecting leavening gas and water vapor as they are evolved (3,4). If the number of nucleating sites is small, then individual cells will tend to be large and the cake to be open-grained. Conversely, with a large number of sites available to be inflated, the cells will be small and the grain fine or close. Since it has been shown[2] that bubble sizes in thick-walled foams follow a single log-normal distribution, average bubble size should be sufficient for predicting the proportion of bubbles which will become critical.

If the expressions for F_3 and F_4 are equated and the expression solved for σ, it is found that the terminal rising velocity of a bubble is

$$\sigma = 2gr^2(\rho_2 - \rho_1)/9\eta \tag{6}$$

Equation 6 shows that, in a liquid of given viscosity, the rate of rise of a bubble due to its bouyancy is proportional to the square of the bubble radius and is inversely proportional to the viscosity. These relationships show that a foam composed of small bubbles suspended in a viscous Newtonian fluid would be more stable with respect to bouyancy-induced segregation than a foam composed of larger bubbles or a foam in a less viscous Newtonian fluid.

In cakes, rapidly rising bubbles cause loss of volume and layering (a gradient in cake grain varying from tight at the bottom of the cake to open at the top). Fewer bubbles should reach sufficient size to rise out of the batter during baking when the number in which the leavening gas collects is large than in the case where only a few bubbles are available to serve as nuclei. This is assumed, because, if only a few

[2] Overbeek, J. th. G. Rijksuniversiteit Te Utretcht, Utrecht, Domplein 28, The Netherlands. Private communication.

sites receive all the gas, these sites must each attain relatively larger size. An increase in viscosity should also reduce volume loss by increasing the critical size which a bubble must attain to rise rapidly.

In the case of cake batters, which are neither homogeneous nor Newtonian, the relationship between gas-liquid surface tension and bubble pressure should hold. Our measurements show some bubble-to-bubble variation in surface tension within a cake batter, due apparently to the inhomogeneity of the system. The batter properties governing bubble movement act in a more complicated way, however. These studies show that cake batters are pseudoplastic (apparent viscosity decreases with increasing shear rate or rate of bubble movement), and that they have a small yield value. Equations 7 and 8, below, describe the effect of yield value, and equation 6 will also apply to cake batters if σ is made the proper function of η.

$$F_5 = Yf(r) \tag{7}$$

F_5 = force which must be exceeded for bubble movement;
Y = yield value;
$f(r)$ = an unspecified function of the bubble radius.

If the expressions for F_3 and F_5 are equated, the boundary condition for no bubble movement is given by the expression:

$$4/3\pi r^3 g(\rho_2 - \rho_1) = Yf(r) \tag{8}$$

If the bubbles which increase in size during the microscopic observation period are taken to be the available nucleating sites, it can be seen that the number increases from unemulsified to the mono- and diglyceride shortening and increases again from that shortening to the high-aerating shortening. Table II shows that average specific volumes obtained increase at the same time the number of nucleating sites increases. It can be argued that the specific volume differences between the cakes made with high-aerating shortening and those made with shortening containing mono- and diglycerides are due to the difference in amount of air incorporated during mixing, with this air serving to augment the gas generated by the leavening system. However, since batters prepared with the unemulsified shortening and those prepared with the mono- and diglyceride shortening had similar specific gravities (Table II), it must be concluded that more of the leavening gas escaped from the unemulsified-shortening batters, presumably by the mechanism described above.

The fact that leavening gas is evolved only into relatively low-pressure bubbles, when they are present, is adequately demonstrated by the photomicrographs in Fig. 1. Neither leavening system nor batter

TABLE II

PHYSICAL CHARACTERISTICS OF BATTERS AND FRACTIONS AND EVALUATIONS OF CAKES PREPARED FROM THEM

VARIANT FORMULA (SHORTENING)	VISCOSITY [a]	YIELD VALUE	SURFACE TENSION OF AQUEOUS FLUID (BUBBLE WALL)	INTERFACIAL TENSION (FAT VS. WATER)	BATTER SPEC. GRAVITY	CAKE		
						Spec. Vol.	Conformation	Texture and Grain
		dynes/cm²						
Unemulsified								
Whole egg	37	10.5	55	5.6	0.93	2.7	34–34–35	Tight—sl. coarse
White	72	26	69	12.9	0.93	2.4	29–32–28	Tight—sl. coarse
Wh + lecithin	39	2	52	2.3	1.06	2.3	28–30–28	Tight—sl. coarse
Mono- and diglyceride								
Whole egg	70	39	46	0.7	0.92	3.1	39–40–39	Fairly open, med. fine
White	83	28	50	0.7	0.94	2.9	35–38–36	Fairly open, fine
Wh. + lecithin	56	10	45	0.6	0.94	2.8	33–36–33	Open—med. fine
High-Aerating								
Whole egg	67	32	45	0.5	0.77	3.2	41–41–41	Open—med. fine
White	160	75	55	0.8	0.75	2.7	35–27–35	Open—coarse
Wh. + lecithin	152	85	64	0.6	0.75	3.1	38–38–37	Open—fine
St. error of measurement	$s \leq 2$	$s \leq 4$	$s \leq 1.5$	$s \leq 10\%$	$s \leq 0.02$	$s \leq 0.03$		

[a] Centipoise at a low rate of shear of 2 sec.$^{-1}$

temperature varied in the test, so it is safe to assume that about the same amount of leavening gas was evolved during the observation period of each series of photomicrographs. In spite of this, the small (high-pressure) bubbles in the emulsified shortening batters shrank. The growth of the larger bubbles had to be from leavening and diffusion of gas from the small bubbles. The tendency of leavening gas to seek the larger bubbles suggests that the release of shortening-entrained bubbles during baking (3) should provide few nuclei for new cells, since the newly released bubbles would be smaller and have higher internal pressure than those which had been growing longer in the aqueous phase. It is possible, however, that in batters with extremely few effective nuclei, all originally active bubbles will escape and nucleation of the final structure will come from entrained bubbles.

Interfacial Tension Effects. The emulsified shortenings yielded much lower fat vs. aqueous interfacial tensions (Table II), and this was accompanied by much less diffusion inhibition. Presumably, dispersibility was increased with the lower surface tensions resulting in smaller fat units, which did not present a sufficient barrier to block diffusion. Neither gas-liquid nor liquid-liquid interfacial tension seemed to affect significantly the amount of air incorporated in a batter during mixing, since the specific gravities of the unemulsified-shortening batters and those made with shortening containing mono- and diglycerides were approximately the same, whereas their liquid-liquid interfacial tensions were markedly different (Table II). The lack of correlation is also shown by the fact that the high-aerating shortening caused a great deal more air incorporation (lower batter specific gravities) than did the mono- and diglyceride shortening, even though their interfacial tension effects were similar. No correlation was observed between the gas-liquid interfacial tension and the amount of air incorporated into the batter during mixing.

Rheological Effects. From the discussion above, it should follow that higher volume and less layering should be obtained with the cakes having high yield values and viscosities. In the experimental cakes prepared in this study (Fig. 2 and Table II), no instance was observed in which an increase in specific volume could be attributed to a change in viscosity or yield value. In the case of C3, Fig. 2, layering was markedly reduced over that in any of the other high-volume cakes, B1, B2, or C1. Since C3 had a yield value more than double that of any of the other high-volume cakes, it is probable that a relationship existed. Apparently, to demonstrate definitely the influence of rheological characteristics, it would be necessary to produce much greater variations without seriously altering the other batter properties.

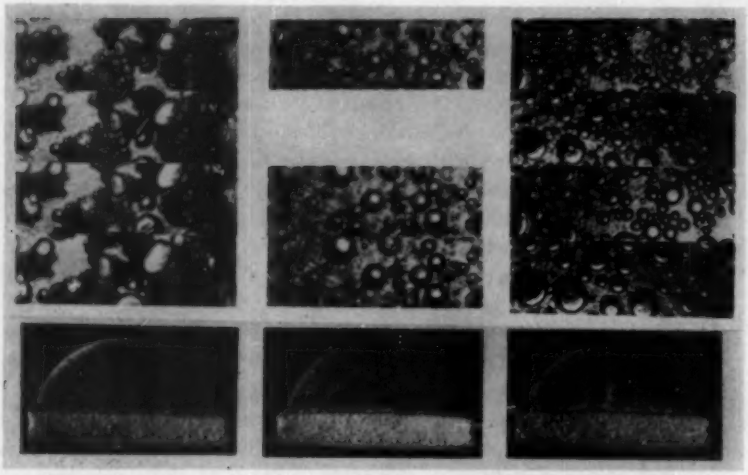

Fig. 1. Photomicrographs of batter distribution and bubble growth in whole-egg cake batters at 25°C.; approx. 150 times actual size. Holding time, top to bottom strips: 0, 15, 45, and 90 minutes. Shortening, left to right: unemulsified; mono- and diglycerides and lecithin; high-aerating. The resulting cakes (respectively) are shown below.

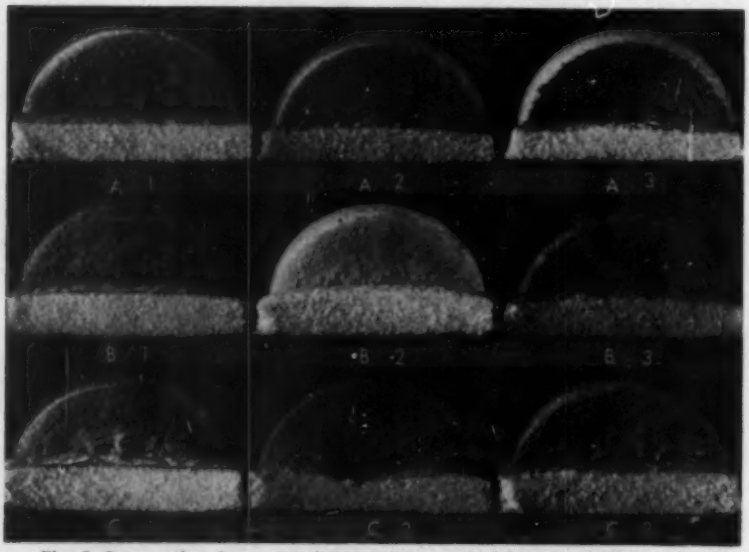

Fig. 2. Layer cakes from experimental batters. A1,2,3, unemulsified shortening; B1,2,3, shortening emulsified with mono- and diglycerides and lecithin; C1,2,3, high-aerating shortening. A1, B1, C1, whole-egg; A2, B2, C2, egg white only; A3, B3, C3, egg white plus lecithin.

If f(r) in equation 7 were specified, equation 8 could be solved for the maximum size of stationary bubbles. However, to the authors' knowledge, this function has not been specified. Although yield values should have the same effect of stopping bubble movement as would very high viscosities, no case was observed in which restriction of bubble movement could be attributed to yield value.

On standing, the bubbles in cake batters appear to increase in size, even if no new leavening gas is evolved. The mechanics of this effect are described below, because they clarify some of the observations in this study. The following equations describe the interaction of two bubbles of radii r_1 and r_2 in a thick-walled foam.

$$dV/dt = -DA\ dC/dX \tag{9}$$

$$P = CK \tag{10}$$

$$dC/dX = (C_2 - C_1)/X \tag{11}$$

In these expressions:

V = ml. of gas;
t = time in seconds;
D = diffusion constant;
A = area normal to the concentration gradient;
C = concentration of the dissolved gas;
X = effective distance on the concentration gradient;
K = Henry's law constant.

If the expression for pressure in equation 5 is substituted in equation 10 (Henry's law, 5), and the resulting expression solved for C, it is found that

$$C = 2\gamma/rK \tag{12}$$

Substituting the expression for C given in equation 12 in the assumed concentration gradient, equation 11, the concentration gradient in terms of surface tension, bubble radii, and Henry's law constant becomes:

$$dC/dX = 2\gamma(r_1 - r_2)/r_1r_2XK \tag{13}$$

Using the description of the concentration gradient in equation 13 in the Fick equation 9 (5), it is found that the rate of diffusion at bubble 2,

$$dV/dt = 2\gamma D\pi r_2(r_2 - r_1)/KXr_1 \tag{14}$$

From equation 14, it is apparent that diffusion will take place toward the larger bubble (dV/dt is positive if r_2 is greater than r_1 and negative if r_1 is larger). The general tendency described more or less exactly by equation 14 is intuitively apparent from a consideration of the following. From equation 5, pressure is higher in smaller bubbles,

and, from equation 10, solubility increases with increasing pressure. The equilibrium concentration of the dissolved gas in the fluid around a small bubble, then, is higher than that surrounding a larger bubble in the same fluid. The dissolved gas, in moving from the region of high concentration to that of low concentration, raises the concentration around the large bubble above the level in equilibrium with the internal pressure of the bubble and comes out of solution into the bubble. This, in turn, makes F_1 exceed F_2 (equations 1 and 2), and that bubble expands to restore the balance, with the net result that the larger bubble's size is increased and pressure is further decreased. Meanwhile, the reduction in concentration around the small bubble causes more gas to be dissolved, further reducing its size and increasing its internal pressure. It can be seen that the driving force causing gas to move from the smaller to the larger bubble is the internal pressure difference or size difference and becomes increasingly large as the process proceeds. The rate at which this takes place is controlled by the constants, γ, D, K, and X in equation 14.

Equation 14 suggests that cakes prepared from batters which have stood (even if no leavening gas is evolved during standing) should have coarser texture and lower volume if no effects other than bubble-to-bubble diffusion are present. As large bubbles grow and small bubbles shrink and disappear, the number of active nucleating sites is reduced, and the mean size of cells must increase. Consequently, the proportion of bubbles attaining critical buoyancy increases. In this study no attempt was made to measure diffusion constants, but the rate of diffusion could be roughly observed in photomicrographs by following the shrinkage of small bubbles.

Diffusion Effects. The photomicrographs in Fig. 1 show size distributions and room-temperature growth patterns of bubbles in whole-egg cake batters made from the three test shortenings. Diffusion results with every test batter are not shown, because differences were shortening-oriented. The shrinkage of small bubbles and growth of large bubbles predicted by the diffusion equation (eq. 14) are quite evident in the cases of the two emulsified shortenings. In the case of the unemulsified shortening, the smaller bubbles, entrained in the fat, show little size change, while a few of the large bubbles show considerable growth. Apparently, very little bubble-to-bubble diffusion is taking place in this case, and the growth of the few large bubbles stems from the slight leavening activity going on during the period of observation. The fact that only a few of the bubbles change in size may mean that only these have sufficient contact with the aqueous phase to serve as nucleating sites, and the entrained bubbles are inert in this phase of structure

development. In any case, the limited number of nucleating sites in the unemulsified shortening must result in a larger proportion of the bubbles attaining critical buoyancy. This result is predicted by the foregoing discussion and is confirmed by the lower specific volumes obtained with the cakes prepared with the unemulsified shortening (Table II).

The dip in cake C2, Fig. 2, is not satisfactorily explained by the batter properties considered in this study.

Conclusion

Experimental results support the theoretical conclusion that by increasing the number of sites which can receive leavening gas one can reduce the average size of individual bubbles and minimize the numbers which reach a critically buoyant size and rise out of the system, carrying their leavening gas with them. Shortening emulsification can affect this situation by 1) permitting the incorporation of more air (bubbles), or 2) making the fat sufficiently dispersible to avoid screening a large portion of the bubbles and rendering them ineffective as nucleating sites.

There is insufficient variation in air-batter surface tension among the formulation variations examined to implicate this property as a major factor affecting bubble or batter characteristics. Fat-aqueous surface tensions, as measured in this study, do not account for the property of high aeration associated with some emulsifier systems, but they do seem to indicate whether a shortening will disperse sufficiently so as not to shroud bubbles and prevent their acting as nucleating sites.

It is clear that these relationships do not account for all variations in cake performance, but their ability to explain many of the examples cited in this study demands further work along these lines.

Acknowledgment

The authors are indebted to John R. Van Wazer for guidance and advice in the phases of the study involving rheological measurements and interpretations; to Mel Tuvell in problems involving interfacial tension measurements; and to Miss S. J. Aulabaugh and Mr. Jack Wahl in photographic problems.

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COMMUNICATION TO THE EDITOR

Obtaining Damaged Starch Mathematically Rather Than Graphically by the Sandstedt and Mattern Procedure

DEAR SIR:

Sandstedt and Mattern (*Cereal Chem. 37:* 379; 1960) recently proposed a method for the quantitative determination of damaged starch of flour. In this method o/o maltose produced after 1- and 2-hour digestion periods, in the presence of excess amylase, is plotted versus time and the intercept of the straight line with the origin taken as o/o maltose from damaged starch, or o/o damaged starch.

An observation in our laboratory by one of us (Jocelyn Rosen) has led to a simplified procedure for calculating, rather than obtaining graphically, starch damage via the Sandstedt and Mattern method. The development of this modification is as follows:

It may be shown from analytical geometry that the equation of a line through two points (x_1, y_1) and (x_2, y_2) is

$$\frac{y - y_1}{x - x_1} = \frac{y_2 - y_1}{x_2 - x_1}$$

If the x axis of the starch damage curve is time in hours, and the y axis o/o maltose, we may assign the following values:

$(x_1, y_1) = (0, y_1) =$ o/o maltose at 0 hour of digestion
$(x, y) = (1, y) =$ o/o maltose at 1 hour of digestion
$(x_2, y_2) = (2, y_2) =$ o/o maltose at 2 hours of digestion

Substituting these coordinates into the above equation for a straight line and solving, we obtain the relationship:

$$y_1 = 2y - y_2$$

where $y_1 =$ o/o damaged starch
$y =$ o/o maltose after 1 hour digestion
$y_2 =$ o/o maltose after 2 hours digestion

The above relationship can then be used to make a simple calculation for damaged starch, eliminating the need to plot data.

J. G. PONTE, JR.
JOCELYN ROSEN
Research Laboratories
CONTINENTAL BAKING COMPANY
Rye, New York

December 29, 1960

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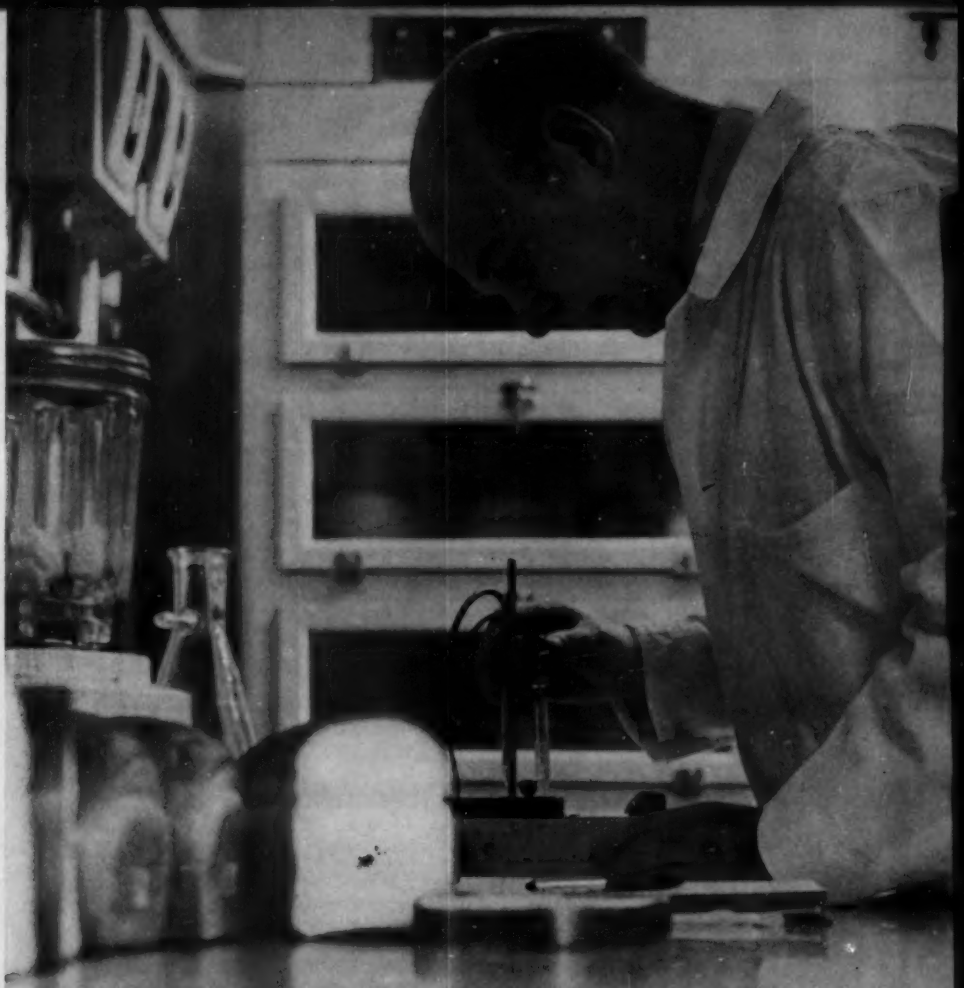

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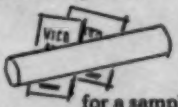

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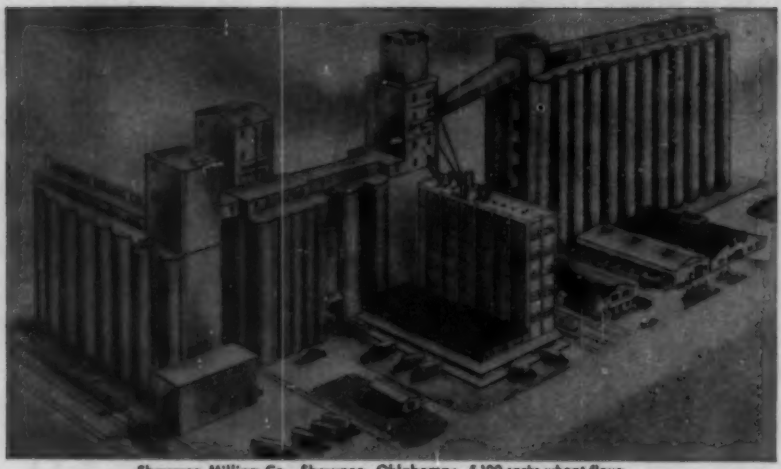
415 WEST SCOTT STREET • CHICAGO 10, ILLINOIS



Shawnee Milling Co., Shawnee, Oklahoma; 5,100 sacks wheat flour; 1,200 sacks corn meal; 250 tons mixed feed; mill elevator storage 2,500,000 bu.

SHAWNEE MILLING COMPANY SCALPS MILLING COSTS

With W&T Flour Treatment

At Shawnee Milling, mill managers control some of their costs with dependable Wallace & Tiernan equipment and processes. They know that quality, cost, and good running time go hand in hand—that they can depend upon W&T to keep its bleaching and maturing processes running —that stoppages won't turn flour into the "dirty" bin for costly reblending.

And Shawnee uses W&T's flour treatment not only for dependability but for performance:

- **Dyox® for fresh, sharp chlorine dioxide gas, made as it is used... precisely metered as a gas not a liquid.**
- **Novadelox® for best color removal, ease of application, consistent results.**
- **Beta Chlora® machines for pinpoint pH control with one, easily-read setting.**

Shawnee Milling Company is only one of the many milling companies using W&T flour treatment. If your mill is not one of these, investigate the advantages of Wallace & Tiernan's complete flour service.

NOVADEL FLOUR SERVICE DIVISION
WALLACE & TIERNAN INCORPORATED
25 MAIN STREET, BELLEVILLE 9, NEW JERSEY
REPRESENTATIVES IN PRINCIPAL CITIES

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